PHYSIOLOGICAL REVIEWS

EDITED FOR

THE AMERICAN PHYSIOLOGICAL SOCIETY

CONTENTS

	PAGE
THE METABOLISM OF THE DEVELOPING EGG. *Joseph Needham*	1
THE PHARMACOLOGY OF ARSPHENAMINE (SALVARSAN) AND RELATED ARSENICALS. *Carl Voegtlin*	63
BACTERIOPHAGE. *Carroll G. Bull*	95
MORPHOGENETIC FACTORS IN THE DIFFERENTIATION OF THE NERVOUS SYSTEM. *C. Judson Herrick*	112

VOL. V, NO. 1

Issued January, 1925

BALTIMORE, U. S. A.

Entered as second-class matter, January 15, 1921, at the Post Office at Baltimore, Md., under the Act of March 8, 1879

Made in United States of America

PHYSIOLOGICAL REVIEWS

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Subscriptions will be received in advance only. The subscription price is:

$6.00 per volume in the United States, net postpaid
$6.25 per volume in Canada, net postpaid
$6.50 per volume elsewhere, net postpaid
$2.50 per single number, net postpaid

Subscriptions should be sent to

D. R. HOOKER, *Managing Editor*
19 W. CHASE STREET, BALTIMORE, MD.

PHYSIOLOGICAL ABSTRACTS

Monthly, price 5s. net. Annual Subscription, post free, 42s.

This Journal is issued by the PHYSIOLOGICAL SOCIETY, acting in co-operation with numerous physiological organisations in Great Britain, America, and other countries. The Editor, Prof. J. MELLANBY, is assisted by a staff of competent abstractors both at home and abroad.

The Journal aims at issuing promptly abstracts of the papers published throughout the world, in physiological and allied sciences (including Plant Physiology).

A number is published at the beginning of each month.

Volume IX began with the April (1924) issue.

Subscriptions are only taken for April to March of the next year, and are payable in advance.

Back numbers, excepting some which are out of print, may be obtained from the Publishers, or any Bookseller. An extra charge is made for some numbers the stock of which is nearly exhausted. Prices on application.

Binding Cases, price 2s. net, are supplied by the Publishers, who can also arrange for binding; prices on application.

PUBLISHED BY

H. K. LEWIS & CO., Ltd.

136 Gower Street LONDON, W. C. 1

Publishing Office, 28 Gower Place, London, W. C. 1.

SUBSCRIPTIONS MAY BE PAID THROUGH ANY BOOKSELLER

WAVERLY PRESS
THE WILLIAMS & WILKINS COMPANY
BALTIMORE, U. S. A.

Medical
Direct
11-29-26
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PHYSIOLOGICAL REVIEWS

VOL. V JANUARY, 1925 No. 1

THE METABOLISM OF THE DEVELOPING EGG

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Among the various departments of biology which have been infiltrated with physico-chemical ideas and terminology during the last half century, embryology has tended, perhaps, to move more slowly than the others toward a completely physico-chemical outlook. It would be possible to adduce many factors to account for this, but one very important one has certainly been the lack of quantitative methods and the presence of certain not very obvious, but yet very real, difficulties inherent in embryological biochemistry.

The literature of this subject is notably characterised by the frame of mind in which research workers have approached its problems. It affords a very interesting example of the way in which a widespread and tacit manner of thought among investigators may sometimes obscure important correlations, and unconsciously hamper real advance. For example, a typical paper on the sugar in the egg will give numerous references to previous work on sugar, but few or none to previous work on eggs. It seems that the egg has been largely used as a source of information about other things much more than as a source of information about itself. As a corollary of this, it follows that, as might be expected, the literature is a difficult one to collect, for data on eggs are frequently found hidden away in a paper dealing with some metabolite, such as creatine. The writer has simply used the eggs as material for creatine experiments, and was not interested in the egg quâ egg. The consequence of this curious orientation of outlook among research workers has been that the literature exhibits a total lack of coördination. The data of many writers, themselves wholly ignorant of one another's papers, fit in wonderfully, in many instances, and throw a flood of light on the processes of embryonic development. The only previous reviews on this subject are that of Preyer (228), who attempted to survey the literature in 1885, but could do little at that time, and

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those of Grafe (102) and Paetchner (212) who in 1910 brought only a few of the facts together. A great deal more work has been done since then.

The manner in which so many workers have endeavoured to throw light on their own problems by examining the behaviour of their substances in the egg as it develops, leads one naturally to ask oneself a question of fundamental importance. How far can we consider processes going on in the embryo likely also to go on in the adult? At first sight we might suppose that if a certain synthesis takes place in the embryo, it will be sure to do so also in the adult. But some hesitation in accepting such a rule as general is undoubtedly necessary. Pembrey (217) showed that the embryo in the hen's egg was not a warm-blooded animal till the very end of the incubation period. His paper will be dealt with in detail later as also will those of Murphy (197) who discovered that the embryo of the hen, unlike the adult, has no resistance to implanted grafts of neoplasm tissue. In the face of evidence such as this it is difficult to believe that one can always argue direct from embryo to full-grown animal. There is evidence, moreover, that as development proceeds, enzymes become active which at the beginning were not so, and that others disappear. Many instances of this will be mentioned later, but as a single example we may cite Herlitzka (121) who found that no peroxidase was demonstrable in the frog's embryo till the twentieth day and no diastase before the ninth. Moreover, certain of the concentration curves of metabolites during development seem to show that enzymes may be absent or inactive at certain stages. In Tomita's (280) curve for lactic acid in the hen's egg, for instance—specially trustworthy owing to the large numbers of eggs he used for his analyses—there is seen a distinct maximum about the fifth day, the concentration rising quickly to it, and rapidly falling off. Certainly not the least plausible explanation for this is that the enzyme which decomposes lactic acid is absent or present in too small quantity to cope with the supply till after the fifth day.

Thus it would seem that, at any rate for the present, we shall be justified in using great caution in arguing directly from embryo to adult. We shall have to conclude that if a synthesis be shown to take place in the embryo, it will not necessarily follow that it probably does so also in the adult. But how far chemical processes are influenced and changed by the conditions of active growth and differentiation found in embryonic life, is indeed the fundamental problem of embryological biochemistry, and must await a somewhat distant future for its complete solution.

For it is true that the difficulties of this work are numerous and perplexing. In the first place, we are still much in the dark as to the constitution of some of the more important constituents of the egg. In spite of much work on the subject (1), (2), (3), (4), (5), (208), (209), (210), we still lack really exact knowledge of the proteins of the yolk, because of the great difficulty of preparing them pure. The membranes of the spaces have been little investigated, except for Riddle (235) who found that the yolk-sac membrane lost in protein and gained in fat after the seventeenth day, and Mömer (191), Abderhalden and Ebstein (2), Abderhalden and Strauss (5) and Buchtala (39), who all investigated the amino-acid content of this protein. It is only necessary to read the literature of "haematogen," for example, to get an idea of the quantity of the work done and the lack of resulting certainty. The egg-lipoids, again, have received much less attention than those from other sources, and until our knowledge of the "static" chemistry of the egg becomes more exact, we shall have to continue to use the most extreme caution in interpreting the changes undergone by measurable metabolites during incubation.

Secondly, it is a commonplace of the poultry farmer that eggs do not develop synchronously, the chances are all against two synchronously laid eggs developing together. Out of a batch of fertile hens' eggs some seem to have the start of their companions by twenty-four hours, some, therefore, will hatch out on the twentieth day, the majority on the twenty-first and occasionally one on the twenty-second. Clearly, this difficulty can only be overcome statistically by taking a sufficient number of eggs, say twenty for every point on a curve. But few of the earlier workers recognised this, and not all present-day ones do either, so that considerable attention has to be paid to this point in drawing conclusions from papers. To take half a dozen eggs and weigh their various fractions to five places of decimals, as the early workers did, is surely just what not to do, if any real understanding of the developmental process is to be arrived at.

A third difficulty which is more subtle than the former two is due to the indefiniteness of morphological boundaries. In cases where embryo and remainder alone are being analysed, should the membranes and allantoic fluid of the embryo be counted as belonging to it or to the rest of the egg? Again, when the yolk-sac is being withdrawn into the body of the chick after the eighteenth day, where does "yolk" end and "intestinal contents" begin? In separating white from shell; should the shell-membrane be analysed with the egg or removed with the shell?

In some cases it may make a good deal of difference, and as many workers fail to say what they did, confusion enters at once. The only remedy is to adhere strictly to well thought out arbitrary boundaries, and to state, when describing work done, exactly what the procedure was with regard to these matters. The same remark applies to the conditions of incubation, i.e., Temperature and Humidity.

In any case, however, the correlation of chemical with morphological fact is very difficult. The data of classical embryology are not very useful for our purpose because the majority of embryologists have been histologists and for them the major interest has lain in the first few hours of development. The Primitive Streak, the Neural Groove; these were the kind of phenomena they preferred to study—after the tenth day the embryo ceased to interest them, it became anatomical. As a result of this, we have almost no information as to how the weights of the various organs change toward the end of development, although it is, indeed, almost exclusively during the last week that the greatest chemical changes occur. Plimmer's and Carpiaux's curves show how constant the phosphorus fractions are till the last week of development. Until we know more about the end of incubation from the morphological side, we cannot do much in the way of correlation.

These, then, are some of the chief difficulties in the path of biochemical embryology. But the rewards of such study seem worth the trouble without a doubt. The avian egg is a closed system for twenty-one days; it loses about 3 liters of CO_2 and a good deal of water, but no volatile nitrogenous compounds; it absorbs oxygen. During that time, from the simple yolk and white of the egg-interior, there evolves the complete chick; with its hemoglobin, its feather-pigments and its multifarious tissue-constituents. The transformation is perhaps more dramatic than any other process in nature, if we except the metamorphosis of insects. And the difficulties are not insuperable. Moreover, bacteria, which in the adult animal "line every natural approach" are absent or almost so from the fertile egg. (See Pennington (218), Baumgarte (21) and Rettger (234).) No food or excreta have to be analysed. We deal with a closed system.

Logically, this review should begin with the process of fertilisation, proceeding afterwards to the changes occurring in the egg as it develops. But the first of these subjects is rather specialised and has already been reviewed by Lillie (166) so here we shall omit it, and use the already fertilised egg as our starting point.

CHEMICAL CHANGES OCCURRING DURING THE EARLY PERIODS OF CELL-DIVISION. Needless to say, we have not yet at our disposal estimation methods sufficiently delicate to enable us to find out much directly about the embryo in its first few hours of development. We may possibly hope that microchemical methods united with a tissue-culture technique will ultimately succeed in throwing some light in this direction. But up to the present what illumination we have we owe to indirect methods of approach. In 1904 Lyon (171) when studying the effect of KCN on segmentation became convinced that there existed a stage 10 to 15 minutes after fertilisation when the egg was peculiarly susceptible to KCN poisoning. The resistance steadily decreased to this point and rose steadily but more slowly away from it. He was able to correlate this with the cleavages as shown in diagram 1. Experiments with hydrogen showed that ten to fifteen minutes after fertilisation a process took place for which oxygen was essential; and contrary to his expectation it seemed that the resting stages were the susceptible ones. In a later paper (172) he found that the resistance to heat also followed a rhythmic course, only in this case the rhythm was reversed and the susceptible periods for KCN were the resistant ones for heat. Cold affected the eggs in the same way as lack of oxygen, except that the resistant periods came a little earlier. As a preliminary result he also stated that the curve for the production of CO_2 seemed to have periodic maxima corresponding to the cleavages; but this has not been confirmed. Gray (103) has since produced evidence against it. Masing (paper untraceable) found that the nuclein content of Arbacia eggs was unchanged from the morula to the blastula stage. Loeb had reported that the nuclein was increased, however, and the whole question needs reinvestigation, especially as Shackell in 1912 (254) found no change in water and alcohol-soluble phosphorus.

Rhythmic changes in sensitivity to HCl had been previously found by Spaulding (262) and in viscosity by Ödquist (205), the former on Sphaerechinus and the latter on Rana fusca eggs—but the most important paper on the subject is that of Herlant in 1920 (120). Herlant, regarding karyokinesis as the result rather than the cause of cell-division, set to work to examine the resting-phases as well as the active ones. By a series of experiments on Sphaerechinus and Paracentrotus eggs he found that plasmolysis could be used as an index of high membrane permeability; and that an alternation of high and low membrane permeability appeared to take place after fertilisation. Diagram 2 represents the conclusions of a very large number of experiments.

The point *A* represents the moment of fertilisation. Following the diagram along its single time axis, the lines *F* and *G* represent the points when the two blastomeres are formed from one cell, i.e., the point of actual cell-division. The periods *B–C* and *D–E* represent the begining and end of the first two mitotic periods. The shaded spaces, from *X* to *Y*, from *F* to *C*, and from *G* to *E*, represent periods in which Herlant found that the cell was permeable to substances dissolved in water. In these periods there was a greatly raised susceptibility to salts, bases and acids; the condition was one which Herlant termed "permeabilité physiologique," because physiologically necessary substances could then enter the cell. On the other hand, the periods *Y–B*, *B–F*, *C–D*, and *D–G*, are periods in which the cell is only susceptible to "lipoid-soluble substances," such as chloral, acetone, saponin and digitonin. There is thus an obvious alternation of properties of the membrane. The period *A–X*, immediately after fertilisation, retains the characteristics of the unfertilised egg, and is more susceptible to ether and chloroform plasmolysis than to water-soluble substances; Herlant considered it as a lag in the development of the new properties.

It is interesting to note how well these results agree with those of Lyon. He had found that the periods of greatest susceptibility to KCN came after fertilisation and regularly after each cleavage. On Herlant's diagram, this exactly corresponds to the periods *F–C* and *G–E*, when the cell is particularly permeable to water-soluble substances. Herlant also observed the same period after fertilisation and before any mitotic processes had taken place. Herlant pointed out that it might seem as if "l'activation equivaudrait physiologiquement a l'achevement d'un division cellulaire." Bataillon showed later that oxygen was necessary for anaphase and telophase, *B–F* in Herlant's diagram, thus again confirming Lyon. Herlant concluded that Overton had been partially right in his theory of membrane permeability, and put forward as explanation of these changes the view that in the membrane itself, a fatty and a protein phase were alternately dispersed and continuous.

Later, in 1922 Fauré-Frémiet (78) investigated the rhythmic changes of the resistance of the sea-urchin egg to cytolysis with mixtures of alcohol, chloroform and ether. After two to two and a half hours the rhythm was lost. His results, shown in figure 1, support those of Herlant, but he does not consider it necessary to assume that a fat and a protein phase in the membrane are alternately dispersed and continuous; it is sufficient to picture more and less fat distributed between the phases at the different periods.

Than this, there is very little more known at present about the chemical events of the first few hours of development. The respiratory quotient would probably render valuable information if it could be determined accurately enough in some micro-respirometer. But the chemical approach at this stage is extremely difficult, for small sea-eggs—necessary because of the comparatively large amounts required for analysis—cannot be timed as to degree of development to within plus or minus an hour. Moreover, in view of the fact that, as we shall see later, the development of fish eggs is fundamentally different in many ways from that of avian eggs, we must be prepared to find great differences also in the early stages. Our knowledge of these points is very one-sided and limited at the present time.

CHEMICAL CHANGES DURING LATER DEVELOPMENT. We are here on surer ground, for much more work has been done on this than on the early stages. The material also has been varied, much more so than in that of the preceding section,—though still not varied enough. The egg of the hen, because of its great convenience, has received more attention than any other, but the egg of the silkworm comes a good second (141) because of the industrial necessities of the silkworm trade in Austria, Italy and Japan. Other insect eggs, such as those of bees (115) have also been investigated, though not to any great extent, and the list is virtually completed by the mention of lizard eggs (101), snake eggs (71), (125) and the eggs of carp and trout (100), (273).

General physical and chemical changes. It was natural that among the earliest observations there should be the fact that the hen's egg diminishes in weight by about 5 grams during its incubation. Prevost and Dumas in 1825 (230) carried out careful weighings and proved this point; they discovered also the fact that the diminution is principally at the beginning; the egg loses less and less progressively. In a later paper (230) they found that the weight of the inorganic constituents remained constant: so they concluded that the loss in weight must be due to loss of carbon dioxide. Another classical paper was published by Baudrimont and Martin St. Ange in 1847 (20). Most of their work was concerned with respiration, but they also showed that there were distinct temperature limits for development, as well as an optimum, and that light was not necessary although moisture was. Dr. H. Murray has kindly let me see the results of his researches soon to be published: he has shown that the principal facts governing water-loss is the humidity of the surrounding atmosphere. Many of the earlier workers' results will therefore have to be accepted with caution for they neither

controlled the humidity nor expressed their results in terms of dry weight of embryo.

Not until physical methods had progressed a great deal further could measurements be carried out on such variables as osmotic pressure and electrical resistance. A priori, in such a complicated system as the developing egg, the electrical resistance would hardly be expected to give much assistance, but its variations have been ascertained by Bellini (23) (fig. 2). As the graph shows, the resistance of the albumen rises more or less steadily till the seventh day, after which Bellini's figures stop, while the yolk remains more or less constant, though declining slowly from the seventh day. Unfertile incubated eggs showed no changes. It is difficult to see what this may mean, except that it might indicate a decrease of electrolytes in the white. The same investigator (23) has measured the viscosity of the egg as it developed. Again, unfertile incubated eggs showed no changes. As in the case of electrical resistance, the viscosity of the white slightly increases (fig. 2) while the yolk drops rapidly to the fifth day and thereafter rises again till the end of incubation. The conclusion is that the yolk is gaining water at the expense of the white, and this is, as a matter of fact, exactly what happens (235), (8). But the gain in water proceeds moderately regularly and, as far as is known, is not at all sufficient to account for Bellini's enormous decrease of viscosity at the fifth day. It is possible that changes in water-content may exercise a more profound influence on embryogenesis than would at first sight appear. According to King (143), if the water-content of toad eggs be reduced by mild desiccation, the male ratio is lowered; while if it be raised by the application of dilute acid, the male ratio is raised. It has been suggested by Huxley (136) and others that this may account for the influence of delayed fertilisation upon the sex-ratio (see Hertwig, 123): for an absorption of water from the eggs might go on in the body of the female. But how changes in water-content could influence such processes directly is perhaps a little difficult to understand from a chemical point of view. Huxley's review should be consulted for the literature of this and kindred subjects.

Figures more easy to interpret are those of Bialaszewicz (29), who measured the osmotic pressure by means of the depression of the freezing point method, at the different stages of incubation in the hen's egg (fig. 3). The yolk is at the beginning very much hypertonic to the white but becomes less so as development proceeds; this may perhaps be the cause of the current of water yolkwards referred to above. The chick's

osmotic pressure rises steadily, and the amniotic fluid, which at first is hypertonic to it, becomes hypotonic to it about the eleventh day. The allantoic fluid is moderately steady till the fourteenth day and thereafter drops a great deal and rather suddenly. This change is possibly due to the fact that up till the thirteenth day the products of the chick's renal secretion are passing into the allantoic fluid —after that date they pass into the amniotic space (8). Perhaps the membranes grow round the chick in such a way that just at that point the allantoic fluid is deprived of the chief source of its osmotically active substances, and its osmotic pressure accordingly falls rapidly. However, probably this factor is only one of several, all acting in the same direction. The rise in osmotic pressure on the part of the chick may indicate an increased metabolic vigour, if the concentration of diffusible metabolites is any index of it. But also at this time, as Von Baer (15) showed, ossification is going on, and the ash content of the embryo is rising. Possibly this may also account in part for the rise in osmotic pressure. It is interesting to note that the yolk of the egg at the beginning is considerably hypotonic to hens' blood.

Little is known as to the meaning of the varying specific gravity of unincubated eggs. Mussehl and Halbersleben (200) could only show that it was no indication of viability.

Most interesting of all the more general physico-chemical work, however, is the paper of Aggazzotti (8) on the acidity of the egg in development. Lehmann (160) had found the liquor amnii to be acid after 14 days' development; but Aggazzotti estimated both the hydrogen ion concentration of the various fluids electrometrically, and also the total acidity by titration to neutrality using L-naphthol-phthalein as indicator. In the infertile incubated egg, no change appeared as long as it was sterile. But in the developing egg, the changes were very marked (figs. 4 and 5). Broadly speaking, the yolk is at the beginning acid, and the white alkaline; whereas at the end of development, the position is reversed —the yolk is neutral and slightly alkaline, while the albumen is distinctly acid. It is not altogether easy to explain these changes, but if, as seems is the case, the protein of the egg-white is broken down into amino-acids before being absorbed by the embryo, some correlation should be possible. Aggazzotti, indeed, points out that at the beginning when amino-nitrogen predominates in the albumen it is still alkaline, but that later on, when most of the nitrogen is in the form of non-amino-N, then the white is becoming acid. As for the yolk, the ammonia-N diminishes in it as incubation proceeds, so that its

neutralisation must be due to something else. It is interesting to note that the pH of the yolk indicates a nearer approach to neutrality than is warranted by the total acidity during the last seven days, and this may indicate the coming into play of a buffer action. It is, as a matter of fact, the case that just at that very time there is an enormous increase in the inorganic phosphate. The great majority of this is undoubtedly in the form of calcium phosphate and is concerned with ossification, but some of it probably becomes sodium and potassium phosphates, and may be responsible for this effect. Another interesting point with regard to the trend toward acidity on the part of the white and not the yolk, is the very largely increased CO_2 production of the embryo, after the ninth day (33). As is seen from figure 8, it is increased about forty times during the last eleven days of incubation, and this exactly coincides with the acidity of the albumen. Undoubtedly the albumen would get a great deal more acid by this CO_2 transport were it not for some such buffering action as the phosphate and protein must exert. The amniotic and allantoic fluids are also very interesting in this connection. The latter is fairly constant and does not alter greatly, but the former grows steadily more acid after the twelfth day. Here probably the same process is taking place as was mentioned in connection with the osmotic relationships of these two fluids. Acid products are supposed to appear in the chick's urine about the eleventh day, and this probably coincides with the first functioning of the kidney—they are emptied into the amniotic space and it becomes acid in consequence. The mesonephros is supposed to be functionless. Pigorini (309) measured the acidity of ether extracts of silkworm eggs and found an increase followed by a general decrease.

There is no doubt that a very great deal can be done by the application of purely physical methods to the egg and such results may be of the greatest value in interpreting chemical data.

The fact that an optimum temperature was necessary for development was observed as far back as 1749 by Réaumur (233).

But the heat-production of the developing egg of the hen was not investigated till 1903. Bohr and Hasselbalch (33) and Langworthy (157) in 1903 obtained figures from which the curve in figure 6 has been constructed. It is interesting, though not unexpected, that it follows pretty closely the curves for CO_2-production and embryo-weight. What was not looked for was the initial absorption of warmth, proceeding in the first three days. What the endothermic processes can be which give rise to this effect, it is very difficult to see; they are completely overshadowed by the exothermic ones after the first three days.

The influence of light on development is a subject upon which all work has ceased since 1880. Higginbotham (124) and Macdonnell (174) were the first workers. The subject was much studied by Yung (306) who used frog, snail and cephalopod eggs. When illuminated with coloured lights, the eggs could develop fastest in the following order:

	days
Violet	17
Blue	19
Yellow	25
White	27
Black	33
Red	36
Green	40

Normal development time 29 days.

This work was confirmed by Schenck (248) and Fatigati (77) the latter of whom used infusorians. As far as the hen is concerned, darkness and light were shown to be immaterial by many of the early workers.

Rusconi (242) reported that the development of frogs' eggs was hastened by passing a current through them; and Maggiorani (175) said that eggs placed in a magnetic field died. These observations have received no confirmation and are only of historical interest. The position in which the hen's egg was incubated was shown to be immaterial by Pflüger (221).

Respiration. Strange though it seems at the present day, it was actually believed at the beginning of the last century that the incubating egg could lead an anaerobic existence without difficulty. Between 1818 and 1840, Erman (74), Viborg (292) and Towne (285) all affirmed that development could proceed normally when the egg was placed in irrespirable gases, or completely immersed in oil. There was considerable controversy about the point, in spite of the experiments of Geoffroy St. Hilaire in 1820 (243) and the inaugural dissertation of Theodore Schwann in 1834 (251) "De Neccessitate äeris atmospherici ad evolutionem pulli in ovo incubito," who used hydrogen. The matter was finally set at rest by the experiments of Dareste (58), Dusing (68), Gerlach (91) and Marshall (178). Baudrimont and St. Ange (20) found that frog's eggs would not develop in air-free or carbon-dioxide-rich water. But the former workers made use largely of varnishes and by painting over eggs with them were able to show without any doubt the necessity of atmospheric oxygen. Their work is fully reviewed by Preyer (228), who himself established the fact that the egg respired long before lungs had appeared in the chick.

It was Baudrimont and St. Ange who first suggested the possibility that the air-space might have importance in this connection, and their work was continued by Dareste. It was found that if the shell over the air-space alone was varnished, the embryos died, just as they did when the whole egg was varnished, but that if all the egg except the air-space was varnished, the embryos developed normally. Fabricius ab Aquapendente had already in the sixteenth century observed that the air-space contained air, and William Harvey also mentions the fact (111). It appeared then that the embryo could only breathe through the air-space. Aqueous transpiration was found to take place all over, but gaseous exchange only by way of the allantoic membrane and the air-space. The contents of the air-space had previously been analysed both by Bischof (31) and by Dulk (66), but their eggs were few, and with the apparatus then at their command (1823) probably their figures were not very reliable. It was left for Aggazzotti in 1914 (9) to go over the same ground with the aid of modern accurate methods, and to examine the air in the air-space at the different stages of development (fig. 7). The percentage of CO_2 remained constant till the sixth day and afterwards rose rapidly, whereas the oxygen percentage began to drop steadily after the tenth day only. The CO_2 is initially high as compared to the atmosphere, but the oxygen is about the same (Aggazzotti) or slightly higher (Hasselbalch). In infertile eggs the only change that occurred was that the CO_2 dropped quickly to atmospheric level, and there remained indefinitely. The reason for the high initial CO_2 content would seem to be that the egg starts with the same CO_2 pressure as the hen's tissues and that if no development begins, the excess of CO_2 simply diffuses out into the air. In the same paper Aggazzotti discusses the effects of taking eggs up to high altitudes and letting them develop there. He found they suffered from partial acapnia and anoxemia, and that this resulted in a higher mortality than among normal eggs. In this connection, Wesselkin (298) studied the resistance of developing hens' eggs to oxygen-lack. He found that 5 per cent oxygen killed the embryo in 48 hours but that it would live for about 70 hours in 12 per cent O_2. He found many deformities were produced by that treatment.

A certain amount of work has also been done upon the resistance of marine eggs to oxygen-want. Loeb (168), Samassa (245), Schultze (249) and Godleffski (97), (98) have all worked along these lines, and the last named concluded that CO_2 has a specifically toxic action on these types of embryo. The only work on the resistance of eggs to

pressure is that of Rauber (232) who found that a pressure of 3 atmospheres killed the chick. Preyer (228) suggested that eggs of mountain and plain birds might differ in this respect: but no one has ever followed out his idea.

It is very interesting to compare Aggazzotti's curves in figure 7 with the figures obtained by Bohr and Hasselbalch for total CO_2 production, shown in figure 8. The first thing which is obvious is that the CO_2-production goes exactly parallel with the embryo-weight, increasing rapidly after the ninth day, though this is probably as much as one would expect. Bakounine (17) suggests that the coincident formation of allantois and H_b is connected with this. But a more interesting fact is that the total CO_2-production increases about forty times during incubation, from 10 to nearly 400 cc. CO_2 per 24 hours, whereas the CO_2 per cent in the air-space increases only four or five times. The only possible conclusion is that the shell and membrane between the air-space and the air, must become progressively more permeable as development proceeds. Now it is surely no unwarrantable proceeding to correlate this with the fact that the shell, as we shall see later, loses 6.1 per cent of its dry weight, and 7.1 per cent of its organic substance to the interior of the egg as development progresses (270). In spite of diligent efforts to find some data as to the histological changes in the shell, no work seems to be discoverable on this subject; Winterstein, for instance (304), only gives the structure of the shell at the beginning. Measurements of the permeability of the shell have only been carried out by Hüfner (133) and Camus (48). The latter contented himself with observing the fact that the weight of the egg increased more after having been boiled than it did after having been placed in cold water. The former, however, did measure the permeability of the shell to gases, but he only took eggs at the beginning of incubation. He found that oxygen passed through least readily and carbon dioxide most readily. The duck's egg was relatively more permeable than that of the hen, and the removal of the shell-membrane produced no change in these general relations. It would be most interesting to repeat this work on the air-space shell during the course of development.

This paper by Bohr and Hasselbalch was the first of a considerable series (33), (34), (35) in which they discovered several facts of great interest. They adduced some evidence that oxygen could act as a stimulus to embryonic growth, and they thought they could detect a definite, though very small, output of gaseous oxygen and nitrogen as the egg developed. This was afterwards completely disproved by Krogh

(151) in an extremely careful research. But one of their more important papers dealt with the respiratory exchange. For the hen's egg, figure 9, it appeared that though on the fifth day the R.Q. was 0.9, it speedily declined there from and remained constant at about 0.71 from the ninth day onwards. The conclusion is that in the very early stages of development, carbohydrate is being utilised as a source of energy, while during the greater part of the time, fat is used. If anything, the high value at the very early stage is too low, for as Mr. J. B. S. Haldane has suggested, the egg will have an alkali reserve sufficient to take up small amounts of CO_2 at early stages. This fits in perfectly with all that we know from the chemical side, not only as regards fat, but also as regards the egg's carbohydrate metabolism. We shall return to this later. The R.Q. of a cold-blooded animal, and rattlesnake egg, was afterwards studied by Bohr (35) and did not vary as did the avian egg. It remained unity during the whole incubation, and this is a fact of great interest when considered in relation to the differences in fat metabolism between eggs of warm- and cold-blooded animals. Moreover, Luciani and Piutti (169) found an R.Q. of 1.0 for Bombyx mori, the silk worm, and Buglia (41) working on the eggs of Aplysia limacina, the sea-hare, found that the respiratory quotient varied not far from unity throughout development. Shearer (256) also found an R.Q. of 0.92 for Echinus eggs. So fat utilisation clearly does not occur in these forms.

The data of Bohr and Hasselbalch for the R.Q. of the chick were afterwards confirmed by Lussanna (170) although he got a slow rise before hatching, which does not appear on their curve. This discrepancy is at present unexplained. Measurements of the respiration of Sabellaria and Arbacia eggs have been made by various workers and on Bombyx mori by Bert (24) and Farkas (76). References will be found in Godleffski's paper.

We may thus put the respiration of the egg in the form of an equation as was done by Baumgartner in 1881 (22).

Weight loss + oxygen uptake = CO_2 given off + water given off.

The oxygen consumption of the chick tissues in vitro has only, it seems, been studied by Shearer (257). He expressed his results in terms of cubic millimeters of oxygen taken up per weight of tissue giving 1 cc. of NH_3 by Kjeldahl estimation. In these units the head of the chick decreased from 15 to 3 from the fifth to the tenth day: while the trunk decreased from about 4 to 2. Acetone powders behaved in the same way; and the conclusion is that as the embryo develops, its metabolism increases in toto, but decreases in intensity per unit value.

Metabolism of proteins. The proteins present in the egg of the hen at the beginning of incubation have been the subject of a good many investigations, but the question is still in a rather unsatisfactory state. While the first few mitoses of the chick are going on, before the egg has left the oviduct of the hen, it is surrounded by a "white" yolk, very different to the yellow yolk of later development. Riddle (235) is the only worker who has analysed the white yolk, and he found it to be more near to ordinary tissue composition than to ordinary yolk.

	PERCENTAGE OF TOTAL SOLIDS					TOTAL PERCENTAGE H_2O
	Phosphatides	Neutral fat	Proteins	Extractives	Ash	
Common fowl yellow yolk.....	21.0	49.2	28.8	0.67	0.83	46.9
Common fowl white yolk.....	11.8	30.0	39.3	5.2	8.82	88.8

The change from white to yellow yolk coincided, says Riddle, with a marked change in growth rate. But in later development it is difficult to be sure of the purity of many of the isolated proteins described in the literature. According to the majority of the evidence, the egg-white contains two proteins, ovoalbumin and ovomucoid, the former a typical albumin and the latter a glucoprotein containing glucose or glucosamine in its molecule. To these Osborne and Campbell (208) add two more, ovomucin and conalbumin, differing to a small degree in each case from the first two. Ovomucin contained twice as much sulphur as any of the others, and conalbumin differed from ovoalbumin in containing no perceptible phosphorus; both these latter were present in smaller quantity than the first-named two. Ovomucoid has been the subject of numerous researches which had for their aim the determination of its sugar content, but their confusion is extreme, and their agreement hardly existent. Mörner (191) found that "perca-extract" appeared to be capable of being used as a specific test for ovomucoid, and by its aid he was able to demonstrate the presence of ovomucoid only in a limited number of species of avian eggs. This would seem to require repetition with another method before complete acceptance is accorded it.

The yolk of the avian egg, according to Plimmer (224), contains three proteins recognised as distinct entities at the present day: Vitellin, Livetin and Gross's protein. The first-named of these has been much investigated and its amino-acid content given by Hugounenq (131), Osborne and Jones (209), Levene and Alsberg (162) and Abderhalden and Hunter (3). It is characterised by a high proportion of glutamic

acid, leucine and proline. Kossel (148) and Milroy (189) had found that vitellin differed from other nucleins in yielding no xanthine bases on hydrolysis. Abderhalden and Hunter drew attention to the existence of some similarity between ovovitellin and casein, both being the "erstern-ährung" of the embryos of oviparous and viviparous animals respectively. According to their figures the resemblance is striking enough but it would not be so much so if for vitellin were taken the averages of the figures obtained by all observers.

	VITELLIN	CASEIN OF COW MILK
Glycine	1.1	0.0
Alanine	Trace	0.9
Amnovalenanic acid	2.4	1.0
Leucine	11.0	10.5
Aspartic acid	0.5	1.2
Glutamic acid	12.2	11.0
Phenylalanine	2.8	3.2
Proline	3.3	3.1
Tyrosine	1.6	4.5

Gross (105) demonstrated that the composition of hen's egg ovovitellin was very close to the icthulin of teleostean eggs. Vitellin, according to many observers, contains also as a prosthetic group, the "hematogen" of Bunge (42) but this substance will receive due consideration under the heading of pigments.

In 1851 Melsens (184) discovered that if CO_2, H_2, N_2, or O_2 were passed through solutions of egg-white, fibrous membranous flakes were formed in the course of half an hour. One per cent was thus transformed, and Gautier (89) was accordingly led to believe in the existence of an ovofibrinogen existing normally in egg-albumen and perhaps associated with an enzyme analogous to thrombin. The evidence for this is not great and it is probable that Melsen's experiment may more easily be explained by the well-known phenomena of coagulation of proteins by light, by shaking, etc.

Peptone, according to Fischel (83), who tested protein-free filtrates by the biuret test, could not be shown to be present in the egg at all till the fifteenth day, but was certainly present on the sixteenth. This does not agree with the work which has been done on non-protein-N as the egg develops and it would be well if it were repeated. Abderhalden and Kempe (4) attempted to throw light on the protein metabolism of the egg by estimating the amounts of glycine, tyrosine and glutamic

acid present at various stages, but they could only show that the total concentration of these substances was unchanged. Levene (161) also showed that free anurio-acids were present during development. Ide (137) and von Furth and Lieben (88) both estimated the tryptophane content of the egg at the beginning of incubation and got quite concordant results.

But now, passing to the dynamic side of the question, what is the first problem that arises if we desire to know something of the manner in which the proteins are metabolised by the developing embryo? An obvious one is the question as to whether there is any preferential absorption of ovoalbumin or of ovomucoid by the chick. This was the original aim of Bywaters in his first paper (45) on this subject. Since ovoalbumin is coagulable by heat and ovomucoid is not, then if there is a preferential absorption, the ratio of uncoagulable to coagulable protein will alter as development progresses. The curve in figure 10 shows that as a matter of fact it keeps quite constant.

DAY	RATIO OF UNCOAGULABLE TO COAGULABLE PROTEIN
0	6.0
1	6.5
3	6.1
5	6.0
7	7.4
11	6.4
13	7 9
18	5.7

The next question was whether there was a splitting off of the carbohydrate molecule from the ovomucoid before absorption. If there was then the ratio of uncoagulable protein expressed as grams N per 100 grams white to the total carbohydrate obtained therefrom expressed as grams glucose per 100 grams white ought to fall. In this case also it remains constant (see fig. 11). Free glucose diminishes fairly rapidly in the white (see under carbohydrate metabolism). Since now the relation of ovoalbumin to ovomucoid remains constant, one of two possibilities may be occurring. Either both proteins are absorbed at the same rate, in the form of their constituent amino-acids or not; or albumin alone is absorbed and fresh albumin is synthesised from ovomucoid as development proceeds. The second alternative is negatived, however, because no free carbohydrate can be found outside the embryo after the first week of incubation. Accordingly there can after that point,

at any rate, be no further breakdown of ovomucoid in the egg-white, though it must be broken down in the embryo. Hirota (126) found an anatomical connection between the amnion and the egg-white appearing after the eleventh day, a "sero-amniotic junction;" and this would certainly aid the absorption of the egg-white proteins. Pott (226) had reported that after the eleventh day of incubation, the egg-white was uncoagulable. All protein-absorption, however, does not go on through allantois, for Riddle (235) found that between the twelfth and nineteenth day, the protein in the yolk increased from 28 to 56 per cent of the total solids; decreasing on the twentieth and twenty-first days to about 38 per cent. Clearly, there is a certain current of protein, as well as water, yolkwards, during the last week of development.

On avian eggs there have been two further extended studies, those of Tomita (281) and Aggazzotti (10), (11). Both concerned themselves with the behavior of the non-protein-N fractions as development proceeded. A paper on technique by Hepburn (118) completes the list for this subject. But Tomita followed the "Reststickstoff" in an attempt to see if it could be correlated with lactic acid production. The curves in figure 12 are constructed from his figures. The total amino-acid-N in the yolk and white are seen to rise gradually all through development, but he did not follow them after the fifteenth day. His figures stand in fairly close agreement with those of Aggazzotti, but not with very great exactness. It will be noticed from his curves that both diamino and monoamino N rise both in yolk and white, but the diamino N rises more than the monoamino. The yolk is also seen to have considerably more total Reststickstoff than the white, a conclusion not in accordance with those of Aggazzotti. From Aggazzotti's curves, figure 13, we must conclude that the changes obtained by Tomita were largely apparent, owing to the varying changes in water-content of the various fractions. When the figures are corrected for this and related to dry weights, the changes are smaller. Nevertheless, they are by no means non-existent, and there can be no doubt that after the eighth day the ammonia-N drops in the albumen, while the free amino-N is higher up to the twelfth day than it is afterwards. In the yolk the changes are all much smaller, but the ammonia-N also drops there after the twelfth day. A continual drying-up of yolk and white, and a regular current of water yolkwards is unmistakable.

As general conclusions, we may say that there is no preferential absorption of ovoalbumin or ovomucoid; that these proteins are broken down to their amino-acids before absorption although it is probable that

the carbohydrate radicle of ovomucoid is not broken down to free glucose before absorption in the later stages and that the variations in N-distribution probably exert a distinct influence on the reactions of the egg-fluids.

When, however, we pass to eggs of cold-blooded animals, the picture is a little different. Gortner (100), (101) has very completely investigated the eggs of the brook-trout, Savellinus fontinalis, and the giant salamander, Cryptobranchus allegheniensis. In both cases he found that there was a constant trend from non-basic N towards basic N (fig. 14). Comparison of figures obtained on eggs of such small size with those obtained on avian eggs is very difficult, because in the former case it is not possible to separate the embryo from the remainder, and the whole egg has to be analysed as one unit. But there were unmistakably profound differences between the two classes, for in both Gortner's types of eggs there was a synthesis of fat in the salamander as much as 14 per cent. As will appear when we come to fat metabolism, this is diametrically opposite to what happens in avian eggs, where fat is undoubtedly used as the energy-source, as is seen from the loss of fat during development and the low R. Q. Tangl and Farkas (273), working on the eggs of the trout, had previously been much surprised at a similar result. In the case of the trout, Gortner calculated that 37 per cent of the energy of development must come from non-protein substances, and of the remaining 63 per cent from proteins, by far the greater portion from monoamino-acids, most of the basic N being retained by the growing organism. Another interesting fact which appeared from the nitrogen distribution was an increase in the ether-soluble N, and if this is any indication of the movements of the lipoid fraction, then there must have been a synthesis of some lecithin-like body. Here again, we have a fundamental difference from the behaviour of avian eggs, as will be very evident when lipoids are dealt with. The avian egg finishes its incubation with about 5 per cent of the lipoids it began with. Gortner in both his cases observed a gain of purine and pyrimidine nitrogen, not unexpected in view of the increase of nuclear matter, and paralleled in the case of the chick.

End-products of protein metabolism do not appear to have received attention worth speaking of. Gori (99), however, found urea in the eggs of Torpedo ocellata and many fish eggs, 0.47 per cent in the former case, and 0.71 per cent as an average in the latter.

Proteolytic enzymes have been found by many observers to be present in the egg. Frankel (85) was not able to find any till after the

seventh day in the hen's eggs, but following that there was an active proteolysis; Wohlgemuth (305), on the other hand, could demonstrate a proteolytic enzyme by the first day. Krukenberg (152), Mroczkoffski (190) and Mieczyslar Halpern (188) all got positive results, but Tallarico's (266) data are of special interest because he obtained a distinct increase of activity as incubation proceeded. Koga, still later (145), has reported that both a trypsin-like and an erepsin-like enzyme is present both in yolk and white.

There can be no doubt that there are enzymes in the egg which can break down proteins and prepare them for embryonic nourishment. These enzymes are probably contained in the egg-white and yolk—though their activity is not set going unless the egg is fertile.

Metabolism of fats. In 1853 Burdach (43), taking as the subject for his inaugural address at Königsberg "De Commutatione substantium proteinacearum in adipem," adduced figures to show that in the snail, Limnaeus stagnalis, other substances were converted into fat during the development of its eggs. From 0.6 the fat-content of an average egg rose to 2.1 grams. But after that time, attention was mostly focussed on to avian eggs and a series of workers, (290), (230) especially Liebermann (165), showed that without any doubt, fat was the most important energy-source of the developing embryo. Pott (226) obtained a decrease of 30 per cent and fat-utilisation is now generally recognised to take place. It was greatly supported by the determination of the R. Q. by Bohr and Hasselbalch, mentioned above. But it became also equally clear (80), (100) that whatever avian eggs might do, marine eggs stored fat without exception and Sommer and Wetzel (260) showed that snake-eggs did too. Accordingly Walter (294) looked to see if there was much carbohydrate in the eggs of the carp, and succeeded in preparing a considerable amount of osazone, while Henze (117) found sugar in octopus eggs. The high R. Q. obtained by Bohr (35) on the snake had pointed the way for a carbohydrate as energy-source. But when Levene (161) attempted to repeat this in the eggs of the cod, and Hammarsten (107) on the eggs of the perch, they were unable to do so and there is still no explanation for this. McClendon (173) could find very little sugar at the beginning of the incubation in the eggs of the giant salamander, although he confirmed Gortner's finding of a synthesis of fat. What then these marine eggs do use as energy-source still remains obscure. It is, of course, possible that they synthesise sugar for energetic purposes in quantities only sufficient for their immediate needs, so that not much would ever be present at one time. Tangl

and Farkas (273) suggested uric acid or urea as the substance needed, though they were not able to demonstrate the presence of either. Gortner (101), however, has made their suggestion useless, because his figures for the distribution of nitrogen leave no room whatever for the large amounts of urea or uric acid which would be needed on the Tangl and Farkas theory. The respiratory quotient of unity certainly suggests the utilisation of a carbohydrate, but it has not been possible so far to demonstrate any polysaccharide, and simple sugar only in a minority of cases, so this question of the energy-source of cold-blooded species' eggs still remains a mystery.

The iodine value of the fat in the avian egg was first investigated in 1903 by Henriques and Hansen (116). They found that fats containing many double bonds were incorporated by the hen into the eggs without change. Mottram (192) later found the iodine value to be fairly constant no matter what the locality or breed, although in some cases the limits of variation were further apart than in others. Incubation, he found, altered the iodine value little, though some breeds showed a peak of desaturation about the eighth day. More important from the dynamic point of view was the paper of Eaves (69). The curves in figure 15, constructed from her figures, show her main result. The iodine value, keeping at first fairly constant both in embryo and remainder, varies greatly after the tenth day. That of the chick suddenly goes up and that of the remainder as suddenly goes down. The former reaches its highest point at the eighteenth day, the latter its lowest at the fifteenth. Thereafter there is a sharp return to about the same value found at the beginning of incubation, and this keeps moderately constant after hatching. What can be the cause of this curious divergence? It is not to be accounted for by the fact that the actual amount of fat is getting less in the remainder and more in the embryo. It looks, indeed, very much as if there was a preferential absorption of the more unsaturated acids by the embryo after the tenth day. Why this should a priori be expected perhaps to be the case will be clear if it is remembered that, according to Bloor and most of the authorities on fat metabolism, desaturation of the fatty acids takes place in the liver before they can be utilised for metabolic purposes. Now it so happens that we have data as to the weights of the liver of the developing embryo during this period, data obtained by Mellanby (183) in quite another connection and for a very different purpose. Figure 16 shows his curve for liver weight. It will be seen that up to the fourteenth day it is exceedingly small, and does not begin to increase till

about that time. Between the fourteenth and twenty-first days, however, it is rising rapidly. If, now, this curve be compared with the iodine value curves of Eaves, it would seem as if it was no unjustifiable hypothesis that the weight of the liver is here the limiting factor. It may perhaps be performing its function of desaturation moderately well when it is very small, but it is possible, nevertheless, that until it has reached a certain size it cannot cope with the work of desaturation offered to it. Accordingly, though both unsaturated and saturated fat may be absorbed by the embryo, only the former will be used, while the latter will be relegated to the yolk until the embryo is in a position to deal with them. A closer investigation of this point might probably yield some interesting information concerning the intimate nature of the process of desaturation in the liver.

In connection with this metabolism of fats, it is very interesting to note that the processes are more or less quiescent until after the eleventh day. Such is the case, as we shall see, with the majority of events in the egg, though there are a few which proceed equally all the way through, and a still smaller number which only happen in the first week. Eaves found, as would be expected, that although the remainder lost regularly its fat at the expense of the chick, yet some was lost in the transfer (see fig. 18). Fat is the most important energy-source of the embryo.

There is possibly a wider biological significance in the synthesis of fat by marine eggs than would at first sight appear. Polimanti (225) who analysed numerous species of fish, found that those known to live mostly just under the surface had greater amounts of fat in them than deep-sea ones. At the same time he drew attention to the fact that Teleostean eggs are known to sink gradually as incubation proceeds. He concluded that they must lose fat, and related this probability to the loss of fat in avian eggs. Unfortunately, the exact reverse is the case with the fishes that have been investigated. Studies on deep-sea and surface eggs in this connection would be of the greatest interest.

Pennington (219), Wohlgemuth (305) and Tallarico (266) all found lipase active in the fresh egg, and the last-named showed that its activity increased as incubation proceeded. Koga (145), going into the matter in more detail, found that the egg possessed both lecithase and monobutyrase. Both were present in white and yolk, but lecithase less in the yolk than in the white and monobutyrase vice versa. It would be very interesting to know more about the activity of these enzymes during the last week of incubation, but Herlitzka, the only investigator who

has followed enzymes through development at all systematically, did not pay any attention to lipolysis.

As regards species differences in fat content of eggs: interesting papers are those of Langworthy (157) and Riddle (235). The latter was able to show a distinct similarity between the common fowl and the jungle fowl, its supposed ancestor, while the common pigeon was noticeably different. The two former have as percentages of total solids 49.2 and 46.7 while the latter has a percentage of 68.6: agreeing more nearly with Langworthy's results for duck, turkey, guineafowl and goose. But this is hardly a metabolic study and their papers should be consulted for the literature.

Metabolism of carbohydrates. The free glucose in the egg has occupied the attention of a great many investigators. A couple of early false alarms were started by Winkler (303) who in 1846 announced the presence of lactose in avian eggs, and later by Weiss (296) who described the isolation of a methylpentose in some quantity. No one has ever been able to repeat these observations and they are now of historical interest only. Since the introduction of accurate and reliable methods of analysis, there has been general agreement that at the beginning of incubation in the hen's egg the amount of glucose in the white is 0.45 per cent and in the yolk 0.2 per cent (218), (119), (146), (247), (279), (1), (57), (7), (32), (70), (149), (156), (159), (176), (196), (204), (212), (246), (253), (263), (307). The evidence for the existence of a polysaccharide is very scanty and what there is is not convincing. Tichomiroff (277) stated that glycogen was present in the eggs of Bombyx mori, Fauré-Fremiet could find none in the frog's egg (78), and Gori (99) by hydrolysing the eggs of Torpedo ocellata, obtained more sugar than could be shown to be there before. Bierry (30) obtained the same result on avian eggs though it took hydrolysis with hydrofluoric acid under pressure to get the effect. If ovomucoid is not broken down under such conditions, it would be somewhat surprising. It seems fairly certain that as a rule no polysaccharide is present.

Both Tomita (278), Satô (247) and Bywaters (47) have obtained figures agreeing very well and showing that although at the beginning of incubation free glucose is present both in yolk and white, it ceases to be so after the tenth day. In figure 17 is shown a curve constructed from the data of Satô and Tomita; Bywater's values are slightly lower, but this is explained by the fact that he used Pavy's (215) method, while they employed the Schenck-Bertrand technique. It is, of course, difficult to draw conclusions easily from the appearance of a curve for a

metabolite such as glucose, which will be bound to have many relationships with other processes, and the general shape of whose curve may hide altogether many minor effects. However, it is possible to draw some general conclusions of no small interest. In the first place, if the curve for the disappearance of sugar in the egg is compared with the respiratory quotient diagram in figure 9 it will be noticed how it corresponds with the high R.Q. observed by Bohr and Hasselbalch in the first five days of development. We cannot escape from the conclusion that carbohydrate metabolism is predominant during the first few days; the time when the shapes of the organs are being laid down—whereas afterwards fat metabolism takes its place almost completely. There are three other correlations which should be noticed at this point. First, it is noticeable that the fat utilisation percentage as shown in figure 18 changes very little during the first five days but that after that time it alters greatly. Secondly, we shall see below that the lactic acid concentration is found to have a remarkably high peak about the fifth day; an indication impossible to overlook that carbohydrate metabolism has its chief importance at the beginning of development. Thirdly, we shall also see when we come to deal with the energetics of development that both the relative and the specific Entwicklungsarbeit are higher in the earlier stages, presumably because the embryo at that time needs more chemical energy for its processes than it does later. The initial heat-absorption during the first three days should also be borne in mind. Tangl (268) has distinguished between "Neubildungsarbeit" and "Wachstumsarbeit," the former being the measure of the work done in laying down new "form;" new spatial arrangements of cells; the latter being the measure of the work done in growth pure and simple, not differentiation and organisation. At present we have possibly just the shadow of a likelihood that carbohydrate metabolism may be associated with Neubildungsarbeit, and fat metabolism with Wachstumsarbeit. If, as Tangl suggests (272), it is legitimate to compare directly the metamorphosis of insects with embryonic growth, and to assume that Neubildungsarbeit alone goes on in the former case, then data concerning the respiratory quotient of insects undergoing metamorphosis would be of great service to us here. What information we have concerning this, however, is contradictory (295).

But these matters will receive further attention in the section on the energetics of embryonic development. It is necessary here to return to the disappearance of glucose as the egg develops. An interesting point in this connection is that in the unfertilised egg there occurs no diminu-

tion in the amount of sugar, but on the other hand a slight increase, due to the loss of water and not real. Presumably there is no enzyme which can hydrolyse glucose in the unfertilised egg; though a glycylotic enzyme has been found in the developing egg by Stepanek (265) and Herlitzka (121). The latter could not demonstrate its presence, however, before the eleventh day, a fact which does not agree with Stepanek or with the early disappearance of sugar, and can possibly be put down to an insufficiently delicate technique.

Tomita (281) in order to study the disappearance of sugar from the egg during incubation made use of an injection technique. Before being placed in the incubator, the egg was painted over at the pointed end with tincture of iodine, and a neat hole drilled in it with a dentist's burr. It was then injected with whatever substance was desired, not more than an actual 0.5 cc. of liquid being used. It was then sealed up by a piece of sticking-plaster and left to incubate. The whole procedure was done under as sterile conditions as possible. In the present writer's experience this method, though very satisfactory on the whole, does not fail to lead to an increased mortality, however great the precautions. Tomita injected in this way from 50 to 400 mgm. of glucose per egg into eggs, and found that the glucose disappeared more rapidly from the yolk than from the white. Tomita also found that the enzyme which produced lactic acid in autolysing eggs from added glucose (280) was much more active in the yolk than in the white.

Lactic acid had been shown to be present in avian eggs by Bonnanni (36) and Anno (13). But the only complete study of the subject is that of Tomita (282). Figure 19 constructed from his figures shows the concentrations of lactic acid present in white and yolk at the different stages of development. The curve is exceedingly interesting, for not only is it practically the only one which shows a peak as early as the fifth day but also, since lactic acid is so intimately related with carbohydrate metabolism, the early peak points to a correlation with the carbohydrate utilisation at the early date. It would be very interesting to know if succinic acid, which had been shown by Moyle (193) to have certain metabolic similarities to lactic acid, also showed a peak at the fifth day. Correlation is not altogether direct and simple for, at any rate in muscle, the process of breakdown of glucose into lactic acid is anaerobic. Nevertheless, the correspondence can hardly be without significance. Moreover, the curve is specially trustworthy for each point on it is an average of about 100 eggs. Tomita was led to believe that the precursor of this lactic acid was glucose rather than alanine,

because of Satô's work, mentioned above, but also because of the fact that when he injected alanine and glucose (281), the former had no effect in increasing the lactic acid maximum, whereas the latter had. Figure 20 shows his results. They left hardly any doubt that the source of the lactic acid is glucose, and accordingly that the whole process is intimately related with the high R.Q. seen at the initial stages of development.

The presence of glucose in eggs seems to be of extreme importance, to judge from experiments which have been done on fasting fishes. Miescher's classical experiments on the Rhine-salmon showed that whatever might happen to the glucose of the body as a whole, it kept a high and constant concentration in the roe. The same thing was shown by Greene (104) for the king-salmon of California, Oncorhyncus tschawytscha. Not the longest fast could alter the glucose concentration of the eggs.

Although, as has been said above, the evidence for the existence of a poly-saccharide in eggs is very scanty and unconvincing, there is yet considerable evidence that a diastatic enzyme is present in eggs. Roger (238), Müller (195), Mueller (194), Diamare (61), Herlitzka (121) and Krukenberg (152) all reported its existence. Koga (145) found in 1923 that the diastase of the fresh hen's egg behaved very like ptyalin for the most part and that it was almost entirely to be found in the yolk; the diastase of the white was thirty-two times less active. Herlitzka found it both in yolk and white from the very beginning, in the hen's egg, but could not find it in the frog's embryo till after the tenth day, although the egg possessed it from the beginning. The presence of diastase when there is no glycogen for it to act on is distinctly odd.

The only investigation on the pentose of the hen's egg during development is that of Mendel and Leavenworth (185). They estimated pentoses by Tollen's method in hen and duck eggs and found a distinct increase from the seventh day onwards, though beginning later in the duck than in the hen (fig. 21). This might be taken to indicate a synthesis of pentose for the increasing quantity of nuclear matter required, but animal nucleic acid, according to the monograph of Jones (139), does not contain a pentose group. The subject, indeed, needs reinvestigation, especially as Mendel and Leavenworth only made a few estimations and did not get a complete curve.

Metabolism of phosphorus. We are, as it would seem, in the happy position of knowing more about the movements of this element in the developing egg than about any other. Moreover, it happens that its

estimation—rather easier than most of the others—is specially useful for we have in phosphorus a key-position, an index, which points to the explanation of several other apparently unrelated phenomena.

The earlier workers contented themselves with estimating the partition of phosphorus in the unincubated egg, and observing the variations that take place on long storage (53), although an inaccurate study on dynamic lives was made by Maxwell (182). It was left for Plimmer and Scott (224) in 1909 to describe in a classical paper the changes which occur as development proceeds. Figure 22 is a series of curves constructed from their data, using the whole egg, unseparated into embryo and remainder. They classified the phosphorus fractions into six classes:

1. Inorganic phosphates soluble in acid water.
2. Organic phosphates soluble in acid water.
3. Phosphatides and related compounds, soluble in ether.
4. Nucleic acid compounds insoluble in water.
5. Vitellin.
6. Total phosphoproteins, remaining after treatment with soda.

Figure 22 is a striking one. A remarkable quiescence is apparent until the fifteenth day, after which time very great changes occur. The ether-soluble (lipoid) phosphorus decreases to about a quarter of its original amount, and there is a corresponding rise in the inorganic phosphate. It is impossible not to believe that the two are related, and the assumption is that the lipoid phosphorus has been broken down to provide inorganic phosphorus, for ossification and other uses. As will be seen in the next section when ossification is under discussion, it is just during this last week of development that that process is going on most vigorously, and the curve for the increase of calcium in the embryo goes almost exactly parallel to the inorganic phosphate curve. This manner of utilisation, however, seems not to be the only one for which increase of inorganic phosphates is needed. It was seen above that the albumen becomes acid toward the end of incubation, and itwas suggested that the almost certain cause is the increased CO_2 transport at the final stages. But although the total CO_2 output increases forty times so that by the nineteenth day no less than 380 cc. of CO_2 are being given off in one twenty-four hour period, yet the albumen never becomes more acid than pH 6.1 although its total titratable acidity is more. A buffer action is indicated. Now reference to figures 23 and 24 also prepared from the data of Plimmer and Scott, shows that the increase of inorganic phosphate is not confined to the embryo. The rise also takes place in

the remainder, though later, so that sodium or potassium phosphate may very likely be the substance responsible for this buffering action. It would be very interesting to know the exact partition of sodium, potassium and calcium in the egg at the end of incubation.

The next thing which is observable from the curves of Plimmer and Scott is the more steady decrease of phosphoprotein, vitellin and water-soluble organic phosphorus. The nuclein P, on the other hand, is the only one which rises, and it does so fairly steadily, indicating, no doubt, a synthesis of nuclear substance. It is not improbable that the vitellin, water-soluble organic P and phosphoprotein are all broken down and partially resynthesised into the nuclein. There seems to be little doubt also that an intermediate stage in one or more of these processes is a water-soluble organic phosphorus compound, for a distinct rise is observable in the water soluble organic phosphorus fraction before it descends. This effect, clearly visible on the diagram for the total egg, is even more obviously present in the chick curves, where the water-soluble phosphorus is initially very high and falls steadily till the end. In this fraction there must be included, presumably, hexose-phosphate if it is present, glycerophosphates and phosphorus compounds of inositol; phytin-like substances. The latter will be returned to under the heading of cycloses. Great interest attaches to these effects from the point of view of bone formation. Robison (237) discovered the presence of an enzyme in calcifying bone, which had the power of breaking down hexose-monophosphoric acid to inorganic phosphate. It also attacked the esters of glycerophosphoric acid but no cyclose phosphorus compounds. It was not present in any other tissue than calcifying bone, except to a slight extent in the kidney. Kay, in unpublished experiments (140), has found that in the hen's egg at the beginning of incubation, there exists practically no substrate for the enzyme, and no enzyme at all. By the twelfth day, however, it is present and very active, together with a considerable amount of substrate. Now, in the curves for chick only (fig. 23) it is noticeable that just during the period of most vigorous bone formation, i.e., from the twelfth to the twenty-first day, it is in the chick that the water-soluble organic phosphorus is decreasing steadily. Taking all the above facts into consideration, the final result seems to be that, as development proceeds, the lipoid phosphorus is transformed into suitable substrate for Robison's bone enzyme, reinforcing thus the small amount of substrate present initially. and then as calcification goes on, is turned into calcium phosphate.

The behaviour of the nuclein phosphorus is also very interesting. In the curves for the whole egg it rises steadily, as has been pointed out above, indicating a synthesis of nuclear matter. But in the curves for the chick alone it rises to a peak on the seventeenth day, thereafter falling till hatching. In the "remainder" curves (fig. 24) it rises more or less steadily. In the first place, this shows that the synthesis of nucleoprotein, by whatever means it is brought about, does not take place only in the chick, it also proceeds outside the chick, and a subsequent absorption must take place. Secondly, the seventeenth day peak in the chick is explained by the fact that the curves are percentage curves and not absolute quantities. After the seventeenth day, the nuclein P in the chick *per cent* of the total P in the chick does not increase, because of the enormous increase of inorganic phosphate after that date. But in absolute quantity it must do, because of the evidence from the curves for the whole egg.

Another possibly significant fact about the curves for the whole egg is the way in which the vitellin P remains constant approximately till the thirteenth day and only decreases after that. In view of the work of Hougounenq (130), which is dealt with under the heading of pigment-formation, this may perhaps be correlated with the formation of hemoglobin. It has been supposed that "hematogen" the precursor of blood pigment, is a prosthetic group of the protein in the vitellin molecule, so that the decrease in vitellin concentration may correspond to the curves of Frankel (86) and Liebermann (165) (fig. 31).

In view of the chemical differences which have been mentioned previously between the embryogenesis of warm- and cold-blooded animals, it is interesting to note that Plimmer and Kaya later confirmed (222) the earlier work of Plimmer and Scott on the frog. They found a decrease in the ether-soluble P and the phosphoprotein P, whereas there was a distinct rise in the concentration of inorganic phosphate. Further, Carpiaux (51) and Masai and Fukutomi (179) have obtained very recently exact confirmation of Plimmer and Scott's results in almost every particular. So there would seem to be little doubt as to the facts and the only difficulty is in their correct interpretation.

The metabolism of calcium. It is here that we meet with the keenest controversy which has taken place in the field of embryological biochemistry. It is true that it never had quite the martial drums and trumpets of "protagon," nor were there ever anything like the number of partisans who flocked to the banners of secretion and filtration in the kidney; nevertheless, it had its polemics and, in point of time, it lasted

as long as "nucleo-histone" itself. The discussion centered around the disputed question as to whether the egg-shell of the avian egg contributed calcium or anything else to the interior during development.

The affair was started by William Prout (231), who in 1822 published in the Philosophical Transactions of the Royal Society "Some experiments on the changes which take place in the fixed principles of the egg during incubation." His figures given here:

	GRAMS PER EGG		
	0 days	11 days	21 days
Phosphoric acid	0.200	0.220	0.210
Earthy carbonates	0.045	0.095	0.195

indicate that the chick at the end of incubation possesses four times as much Ca as the total egg-interior had at the beginning, and the only possible place of origin must be the shell. The question, however, owing to the improvements in technique during the nineteenth century could not be considered as closed, and between 1870 and 1877 Hermann and Voit (122) and Voit (293) came to diametrically opposite conclusions. The latter found that the per cent of Ca in the shell-ash was 52.46 at the beginning and 52.45 on the nineteenth day, so he refused to believe that there had been any loss. Moreover, he found 0.0234 gram Ca in the embryo at the end, and 0.0347 gram in the yolk and white at the beginning: 2.142 grams Ca in the shell at the beginning and 2.157 grams there at the end. In the following year (1878) Grüwe (106) confirmed some of these experiments, but believed that lecithin P was broken down during development and became inorganic P in the shell. His methods were doubtful. But a return was made to Prout's view by Vaughan and Bills (291). They pointed out that to take only twenty eggs or so, as the preceding workers had done, and to weigh the fractions to six places of decimals, was not a sufficiently accurate proceeding. From their own experiments they concluded that Prout had been right and that a transfer of Ca from shell to embryo did undoubtedly take place.

	gram Ca		*grams Ca*
In chick at end	0.382	In shell at beginning	5.685
In yolk and white at beginning	0.070	In shell at end	5.362
Gained from somewhere by chick	0.312	Lost to somewhere by shell	0.323

The matter, however, did not long remain in such a definite state. Pott and Preyer (227), using a large number of eggs, decided that the embryo removes neither calcium nor phosphorus from the shell.

	gram Ca
Ca in interior at beginning	0.124
Ca in chick at end	0.0913

The average Ca in the shell at the beginning was 2.268 and at the end 2.295. Very definitely these authors concluded there was no transfer. It might have been supposed that their long and careful paper would have settled the point, but their results were to be entirely reversed by all the subsequent work. Tangl (270) some years later went into the matter again, using Plymouth hens on a diet standarised with regard to inorganic salts, especially Ca, and taking great care to know the exact time of laying.

	BEGINNING	END	LOSS PER CENT OF SHELL WEIGHT
Organic substance in shell	2.67	2.45	7.1
Dry weight of shell	5.76	5.32	6.1
Ash	3.08	2.87	5.2
Ca	2.13	1.97	6.5

"Ganz deutlich was dafür spricht" he says, "dass wahrend der Bebrutung aus der Eischale organische und anorganische Substanzen unter der letzteren Kalk, verschwinden. Die Eischale beteiligt sich zweifellos am Stoffumsatz des Eiinnern wahrend der Entwicklung des Hühnchens." An average of 150 mgm. of Ca is lost to the interior by the shell as the egg develops. Tangl pointed out that it has long been known that the shell becomes more and more brittle as incubation proceeds and suggested that that might have something to do with the loss of Ca and organic substance which takes place during development. It also cannot fail to be related in some way with the fact that the shell must become more permeable to CO_2 during development, as noted above. But as to how the transport of Ca is brought about, no theory has as yet been suggested; though Dröge (64) and others have thought that a soluble bicarbonate might be effective.

That it does take place, however, was shown by Carpiaux (50) in 1908 and by Plimmer and Lowndes (223) in 1924; figure 25 compiled from their data demonstrates a large increase of calcium in the embryo and a loss of it from the shell. Plimmer and Lowndes also found that

the Ca in the shell-membrane steadily increased till the twentieth day, after which there was a slight diminution.

The only explanation for the great divergence of results which is seen in the early part of this controversy would seem to be the varying accuracy of experimental methods. It is probably safe to believe, however, that we have now arrived at a stage of finality in this respect.

A considerable amount of work has been done on the influence of diet on the hen's capacity for shell-formation. This hardly comes under the province of this review, but it is interesting to note that, according to Buchner, Martin, Pierce and Peter (38), calcium carbonate can be used by the hen both for bone- and shell-formation, whereas calcium phosphate can only be used for bone. Their paper should be referred to for the literature.

The metabolism of sulphur. Of this we know very little. The only early work was that of Liebermann (165) who estimated the sulphur in the feathers of the chick as it developed. His methods were poor and his figures form no definite gradation, so probably little weight should be placed on them.

Hopkins' isolation of glutathione in 1921 (128) rendered it essential that the matter should be reinvestigated. He himself noticed that no nitroprusside test could be obtained from the unincubated egg, but that a brilliant one was given by the third-day embryo. Later, Tunnicliffe (288) attacked the problem, and found that the substance increased in amount pari passu with the weight of the embryo.

Metabolism of lipoids, sterols and cycloses. In all eggs, it would seem, there is a utilisation and decrease of the lecithin group. The work of Plimmer and Scott (224), Plimmer and Kaya (222), Masai and Fukutomi (179), Carpiaux (50), Riddle (235) and Grüwe (106) supports this, and there are only two conflicting points: Gortner (100) found a slight increase of ether-soluble-N as the eggs of the trout developed, and Tichomiroff (277) stated that the lecithin of silkworm eggs increased during incubation. No work whatever has been done on the behaviour of other lipoids than lecithin as the egg develops, so no data are at hand on what would be an extremely interesting matter.

Now, as the lecithin breaks down, what would be expected to arise from it as breakdown products? In the first place, inorganic phosphate, which has received attention above; in the second place, choline, and thirdly, higher fatty acids. In order to understand the interconnections in this matter, it will be necessary at this point to deal with what is known of the metabolism of some of these related substances.

Cholesterol was studied with regard to the egg as early as 1885. Tichomiroff (277) in a series of observations on the embryogenesis of the silkworm, Bombyx mori, found a definite decrease in the free cholesterol of the egg as it developed. He was confirmed by Parke (214) and by Mendel and Leavenworth (185), but none of these observers obtained more than a couple of points from which a curve might be constructed. Ellis and Gardner (72) recorded later some figures which showed that no cholesterol was lost or gained in the hen's egg as it developed; but their data did not indicate whether the free cholesterol rose or fell. Their paper is difficult to understand as they give no indication as to what day of incubation their figures refer to. More important, however, was the work of Hanes (109). His paper is a splendid example of the help that can be afforded to the biochemist by the histologist who uses microchemical methods with understanding. In examining the developing liver of the chick he observed that a change came over it about the fourteenth day rather suddenly. It became bright yellow and microscopically was seen to be full of doubly-refracting droplets. Staining reactions made it clear that they were not composed of neutral fat, but could not be anything else except esters of cholesterol. If warmed to 40°C. they became isotropic, and were very resistant to autolysis. Hanes correlated this with Plimmer and Scott's data concerning the breakdown of lecithin. Surely, he said, the fatty acids set free in that way must be esterified by cholesterol. Windaus (302) had found that in pathological calcification, cholesterol esters are often present, and it had previously been suggested that one of the functions of cholesterol might be the combination with excess of fatty acids produced by lecithin breakdown.

To see if this hypothesis of Hanes could be confirmed chemically was the aim which Mueller (194) set himself. He altogether succeeded in doing so. His curves, shown in figure 26, show distinctly that synthesis of cholesterol esters about the thirteenth day, postulated by Hanes. Free cholesterol, as previously found by Parke and Tichomiroff, falls as development goes on, but is combined with fatty acids, thus not being destroyed. The correspondence between lecithin broken down and cholesterol combined with fatty acid, is not exact, because there exists only about 0.3 gram cholesterol per egg to detoxicate the results of the breakdown of 0.75 gram or more of lecithin; however, most of the fat would probably be metabolised as an energy-source, and the cholesterol mechanism might act as a brake. However that may be, the correlation is very interesting and assuredly real.

Of course, another explanation for the cholesterol changes might be found in the cholesterol-lecithin balance which, according to some workers, seems to be an essential factor in the maintenance of normal conditions (236). Thus we should have to deal with a detoxication of cholesterol rather than a detoxication of excess of fatty acids.

The other principal breakdown product of lecithin would be expected to be choline. We should imagine that the free choline would increase noticeably during development, unless some other mechanism came into play to remove it. Both Sharpe (255) and Okada (206) have studied this problem: Sharpe, thinking that since guanidine increases markedly in the egg during incubation (44) choline should decrease if it was the precursor of guanidine, made a few estimations to see if it did. As is seen in figure 27, he found the total (combined) choline did decrease, and obtained an indication that the free choline increased. But his points were so few that not much weight could be placed on them had it not been for the much completer research of Okada (206). This worker only estimated the free choline and found a perfectly consistent rise, followed by a fall after hatching. There is no proof from these data that choline is a precursor of guanidine, all they show is that lecithin breaks down as incubation proceeds, giving rise to free choline. As a matter of fact, the rise of free choline accounts for practically all the choline that theoretically ought to be produced from the lecithin destroyed, and very little of the choline can have contributed to guanidine synthesis. But Burns' curve, figure 30, shows that it cannot be more than a contributory source and the question is by no means settled yet. The total phosphorus in the egg (224) is 215 mgm. P_2O_5 or 46.9 mgm. P. Of this, lecithin amounts to 65 per cent and consequently the amount of P present as lecithin P is 30.4 mgm. so that the total amount of lecithin in one egg is about 769 mgm. From the breakdown of this quantity of lecithin approximately 0.104 mgm. of choline would be expected: now since the egg weighs about 50 grams, this will be 208 mgm. per cent. Now as is seen from Okada's curve: the highest concentration of choline reached is 190 mgm. per cent; so very little remains for guanidine synthesis. Of course, it is possible that the sudden drop of choline after hatching, may indicate a synthesis of guanidine.

A little work has been done on the presence of cholesterol in marine eggs. Matthews (180) failed to find any cholesterol in the Asteria (starfish egg), though a good deal was present in that of Arbacia (sea-urchin). He correlated this with the greater sensitivity to cytolysis which the former is known to possess. From Asteria eggs he also iso-

lated a phosphatide containing 10 per cent of reducing sugar. This work has not as yet been confirmed. Clowes, Chambers and Page (56) by experiments involving digitonin and the micro-dissection technique, concluded that the cholesterol in small marine eggs was almost entirely localised at their periphery. But this work, though very interesting in relation to the fact that Chambers has located the essential mechanism of fertilisation at the periphery, hardly comes within the scope of this review.

The cycloses remain. The only member of this group occurring in the animal body and of any wide significance is i-Inositol, or hexahydroxycyclohexane. In 1909 Klein (144) failed to find any inositol in the hen's egg at the beginning of incubation, but succeeded in isolating some from the fully developed chick. Using a quantitative estimation method, Needham in 1923 (201) found that the inositol-content of the Black Leghorn hen's egg was about 6 mgm. per cent at the beginning of incubation and rose to nearly 60 at the end. His curves are given in figure 28. It was not surprising that the small amount at the beginning should have been overlooked by Klein, for he only used two eggs and had but a very rough method.

But from what source does the cyclose take its origin? A true synthesis is indicated, for the alternative hypothesis, that it should arise from a phytin-like body, a phosphorus ester of inositol, is impossible. If it did, there would need to be more than three times the amount of water-soluble organic phosphorus at the beginning than there is. In experiments described in a later paper (202) Needham, using Tomita's injection method, injected glucose, sodium phytate and hexamethylenetetramine into fertilised, unincubated hen's eggs. The latter substance breaks down into formaldehyde in the body, and it was thought that formaldehyde might be the precursor of inositol. The phytate and hexamethylenetetramine eggs did not develop after about the third day, but those which had been injected with glucose developed up to the fourteenth day, and then only died as the result of an accident. The result of the injection of glucose was to cause twice the normal amount of inositol to be formed by the seventh day. No doubt could remain but that there had been a synthesis of inositol from glucose, and that probably this normally took place. We have as yet, unfortunately, no idea as to what functions inositol has in metabolism and growth.

Metabolism of purines. Little is known of this subject. Neither allantoin nor uric acid have been estimated during development. The only researches are those of Tichomiroff (277), Mendel and Leavenworth

(185), Kossel (148), Mesernitzky (187). The last-named found that xanthine appeared to exist in the same concentration in the embryo at the end of development as in the egg at the beginning. This paper is in Russian and not available in England, so it is not possible to know whether the reference is to free purine or to combined. Mendel and Leavenworth obtained a rise from 0.002 to 0.029 gram per egg, expressed as total purines, greater in the duck than in the hen, But their figures were very few. Tichomiroff, working on the eggs of Bombyx mori, found an increase of from 0.02 per cent to 0.23 per cent of purine bases, hypoxanthine and xanthine keeping about equal. When Mendel and Leavenworth's figures are reduced to the same expression, they work out at 0.004 to 0.06 per cent, so the agreement is not very good. Kossel found in the hen's egg 0.28 per cent guanine and 0.66 per cent hypoxanthine by the fifteenth day. The whole matter requires reinvestigation.

Metabolism of guanidine and creatine. Salkoffski (244) and Kojo (146) reported that they had been able to isolate creatine from eggs, but the first dynamic study of the substance in the egg was that of Mellanby (183). In the fresh unincubated egg he was unable to find any, but after the fourteenth day it had made its appearance and rose steadily to the end of incubation, except for a slight kink about the nineteenth day, which was also shown by body-weight and by liver-weight. The latter part of the curve was confirmed later by Needham (201) who, however, made no observations on stages earlier than the seventeenth day. His values were in all cases about 4 to 5 mgm. lower than those of Mellanby, but the curves were parallel as is seen in figure 29. According to Mellanby, there is no creatine to be found on the eleventh day, though muscular tissue is then present in some quantity. Mellanby attempted to relate the increasing liver-weight with the increasing creatine-content, but there seems little reason to do so, since a very large number of changes are going on during the last week of incubation, and it is not obvious why the liver should have anything to do with the question, in default of other evidence in favour of it.

Guanidine in the egg was the subject of a paper of Burns (44). As part of an attempt to gain some knowledge of the possible precursors of creatine, he estimated the guanidine content of the egg during incubation, and a curve constructed from his data is given in figure 30. It will be seen that the guanidine content rises steadily and reaches a plateau between the ninth and sixteenth days, after which if falls till the end of incubation. At first sight this fits in excellently with the curves of

Mellanby and Needham for creatinine, and looks as if the guanidine is forming creatinine. But unfortunately Burns' method of estimation included creatine, and the guanidine in his curve is total guanidine. Burns attempted to explain his results by one of two views. In the first place, the protein complex containing guanidine may have given place, by the sixteenth day, to one which is more resistant to oxidation with calcium permanganate, according to his method. Or, secondly, some possible precursor of guanidine, whatever it might be, might have been exhausted by that time. The second explanation can hardly suffice, for it might account for a plateau and no further increase, but it is difficult to see how it can account for a decrease. Nor is the first explanation very plausible. Certainly, if creatine were not destroyed by calcium permanganate oxidation, the curve would be readily explained, but since the contrary is the case, we must await further work for the correlation of this very interesting subject. Of one thing we can be sure, and that is the accuracy of Burns' work; the number of eggs taken was very large indeed, and that, in this kind of work, inspires confidence more than anything else.

Other inorganic substances than phosphorus and calcium. Of the metabolic relationships of these nothing whatever is known:

Arsenic	Gautier (90) Bertrand (25)	Hen 0.01 mgm. (half in white and half in yolk)
Boron	Bertrand and Agulhon (26), (27)	Hen yolk 0.016 mgm. per kilo white 1 mgm. per kilo echinoderm egg +
Copper	Dhéré (59)	Marine eggs 0.5 to 0.8 mgm. per 100 eggs
Fluorine	Tammann (267)	102 grams yolk contained 0.0012 gram hen 84 grams white contained 0.0009
Iodine	Felsenberg (81) Bonnanni (37)	Hen +
Manganese	Bertrand and Medigreceaunu (28)	Hen. None in white, a little in yolk
Sulphur	Liebermann (165)	Hen. Analyses of chick; method doubtful
Iron	Hartung (110)	Hen + food rich in Fe increases it

Pigments. No efforts have so far been made to ascertain what becomes of the lipochromes in the egg as it develops, nor whether the lipochromes of the chick are identical with those of the unincubated egg. It would seem as if there was destruction of lipochrome, from the fact that the chick is not nearly so heavily pigmented as the yolk and Wohlgemuth (305) described a "chromolytic" enzyme. But it is much to be wished that this point be investigated.

A considerable amount of work, however, has been done on the carotin of the fresh egg. It will not be reviewed here as it appertains rather to the static chemistry of the egg; the literature will be found in the paper by Willstatter and Escher (301) and the review by Palmer (213). That lipochromes are not essential for development was shown by Palmer (213), who succeeded in rearing completely many eggs from hens fed on an entirely carotin-free diet. Nor does the vitamin-content of eggs appear to be influenced in the least by this deprivation of carotin.

The subject of the synthesis of hemoglobin has not failed to attract a considerable amount of attention. Figure 31 shows a curve constructed from data given by Frankel (85) and Liebermann (165) and expresses the amount of hemoglobin present at different times of development. Both workers employed a colorimetric technique. Very early in the history of the subject, von Bunge (43) described a substance which he isolated from the unincubated egg and termed "hematogen." It resembled ordinary nuclein, except that it had a Fe content almost as high as hemoglobin.

	NUCLEIN	HEMATOGEN	HEMOGLOBIN
C	40.81	42.11	54.26
H	5.38	6.08	7.10
N	15.98	14.73	16.21
S	0.38	0.55	0.54
P_2O_5	6.19	5.19	(P) 0.77
Fe	0.00	0.29	0.43
O	31.26	31.05	20.69

Hougounenq and Morel (131), (132) prepared Hematogen by first preparing vitellin fairly pure and digesting it with pepsin for two months extracting the whole mass with ether once a week. The residue on this treatment yielded no xanthine bases or reducing sugar on hydrolysis and to this product alone the name "hematogen" was applied by Hougounenq and his collaborators. If this substance was treated with con-

centrated sulphuric acid, there remained as an insoluble residue, a deep brown pigment, which gave no absorption bands and as regards Fe content resembled the product obtained by the action of alkaline persulphates on hematin. The protein part, soluble in concentrated sulphuric acid, and capable of being split off easily from the pigment, had a high content of diamino-acids, and behaved in other ways not unlike a histone. All avian eggs investigated contained this substance, but those of the herring did not, and the eggs of the carp possessed only very little. Hougounenq explained the divergence by saying that marine eggs were not closed systems like those of land-animals, and that consequently they had less need of reserve substance. This argument is not very convincing; one would hardly expect a "hemocyaninogen" to diffuse into them from the sea-water.

In later papers Hougounenq (132) gave figures for analysis of the substances that he isolated—see above.

Hougounenq's Hematogen

C	43.5
H	6.9
N	12.6
S	trace
P_2O_5	8.7
Fe	0.45
O	27.367

It is difficult, however, to say much about the actual formation of hemoglobin in the egg, the only significant fact is that the great rise in hemoglobin content does not seem to begin till after the tenth day. But now it is not till about that time that the vitellin P in the curves of Plimmer and Scott (fig. 22) begins to decrease. It is not impossible that this may be more than a coincidence.

Of the formation of feather-pigments nothing whatever is known. Banta and Gortner (18) found tyrosinase to be present in the eggs of Hyla, Rana and Bufo, though not in Amolyostoma punctatum and Sperlepes bilineatus. The work of Koga (145) throws a little more light on this point. Koga found that he could demonstrate a tyrosinase-like enzyme distinctly at the beginning of incubation in the yolk of the hen's egg but not in the white. The enzyme would not act on tyrosin but quickly blackened solutions of pyrocatechol, adrenalin, and dihydroxyphenylalanine. That there was also a substance in the yolk capable or potentially capable of being transformed into some kind of

melanin, was shown from the fact that he was able to extract from the yolk a substance which was turned deep brown by the oxidase of the fungus Russula delica. So we seem to have indications, if no more, that both enzyme and substrate occur in the yolk of the hen's egg at the beginning of incubation.

Hammarsten (108) stated that the eggs of river-crabs and lobsters contained the same pigment as their chitinous membranes: cyanocrystallin. If the eggs were boiled they went red. Other marine eggs have been investigated by Maly (177) and Sorby (261).

Other changes during development. It must have occurred to more than one worker in these fields from time to time, that possibly not only were easily-estimable compounds synthesised as the egg developed, but also that there was an ontogenetic procession, as it were, of more subtle substances such as enzymes and hormones. Was it not very likely that the egg did not possess initially the full complement of enzymes and hormones belonging to the adult organism, but that these also were synthesised as development progressed, in much the same way as inorganic phosphates or purine bases? There has never failed certain evidence that many if not most of these more subtle agents have a relatively simple molecular structure. But the only researches which were definitely undertaken to prove this point were those of Herlitzka, some of whose observations have been already referred to under the heading of various enzymes.

Nevertheless, there are certain other observations which may best be classified under this heading, and which show that physiologically the egg is a different system in these ways before and after development. The first of these is the work of Pembrey (217) on the poikilothermicity of the egg, and the second that of Murphy (197), (198), (199) on its varying resistance to the implantation of cancer tissue.

As far as enzymes are concerned, Herlitzka (121) made an interesting though incomplete study of the appearance and disappearance of enzymes in the eggs of the hen and frog as development progressed. A table put together from his data is shown on page 41.

It will be seen that he paid chief attention to the various types of oxidising enzymes. Catalase had been found in many types of eggs by Pennington and Robertson (219), Tallarico (266), Rullmann (241) and Rogers and Winternitz (239), the last named of whom had reported that its activity increased in the chick as it developed. Koga (145) alone has been unable to demonstrate it, though he had found salicylase and histozym. But Herlitzka observed that it appeared in the hen embryo

of the second day and the yolk of the fourth day, while in the frog egg it was present all the time. He concluded that the embryo of the hen was responsible for its formation. Diastase and invertase, though present in hen egg yolk at the beginning, could not be discovered in the embryo till the fourth day. No glycolytic enzyme could be discovered in any egg at any time. Herlitzka drew attention to the fact that a synthetic oxidase, the p-phenylene-diamine indophenol oxidase was present in the hen's egg at a very early stage and not afterwards. Just as in the case of catalase, peroxidase appeared to be formed in the embryo only, but unlike the former, it did not pass into the yolk. In the frog, it was noticeable that the appearance of guiacum resin oxidase preceded that of

		LACCASE (HYDROQUINONE OXIDASE)		TYROSINASE		GUAIACOL OXIDASE		GUAIAC RESIN. OXIDASE		PPH. DIAM. L-NAPTL.		PEROXIDASE		CATALASE		DIASTASE		INVERTASE		GLYCOLYSIS	
		Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg	Yolk	Egg
Chick	0	+	−	−	−	−	−	−	−	+	−	−	−	−	−	+	−	+	−	−	−
	2	+	−	−	−	−	−	−	+	−	−	−	+	−	+	+	−	+	−	−	−
	4	+	−	−	−	−	−	−	+	−	−	−	+	+	+	−	+	−	+	−	−
	11	+	+	−	−	−	−	−	+	−	−	−	+	+	+	−	+	−	+	−	−
Frog	0	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	+	−	−	−
	10	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	+	+	+	−	−
	17	−	+	−	+	−	−	−	+	−	−	−	+	+	+	+	+	+	+	−	−
	20	−	+	−	+	−	−	−	+	−	−	+	+	+	+	−	+	−	+	−	−

peroxidase. As a general conclusion from this work, we may be sure that some degree of "ontogenesis of enzyme" does occur, although it would be rash to lay too great stress on the details of Herlitzka's work carried out as it was at a date when accurate methods were not so numerous as they are now.

That enzymes vary in activity during the development of the egg is undoubted: we have already seen indirect proof of it in such changes as happen to lactic acid and lecithin. More direct evidence is afforded by the work of Kay (140), who found that the bone-forming enzyme of Robison (237) was not present at the commencement of incubation. Abelous and Aloy (6) measured the activity of the enzyme which reduces nitrates, at different stages of development, and obtained the curve given in figure 32; nitrate being estimated colorimetrically.

According to Dixon (63) there is evidence that this enzyme is identical with that in milk which reduces methylene blue in the presence of some oxygen-acceptor such as an aldehyde.

Now whether or not enzymes or hormones govern the response of the embryo to changes of external temperature and to implantation of cancer cells, it is an undoubted fact that the egg differs greatly according to stage of incubation in its reaction to these things. Pembrey (217) showed that almost until the last day of development changes in the external temperature determine, after a longer or shorter period, changes in the same direction in the respiratory exchange. But on the last day a fall of 20° in the external environment will, within 15 minutes, raise the CO_2-output to double its previous amount. Before the chick emerges from its shell, the transition occurs. An extended series of experiments showed that the state of cold-bloodedness might or might not pass to one of warm-bloodedness, depending on whether the animal was vigorous and healthy or not. Pembrey concluded that it must depend therefore, on the degree to which there was nervous control of muscles, and hence a power of regulating heat-production. This work is indeed of great interest and it would be a good thing if further attempts could be made to ascertain the exact mechanism of this change.

Even more subtle is the mechanism revealed by the work of Murphy. In the first paper (197) he pointed out that the avian embryo has no means of defence against the growth of tissues of foreign species. Rat tumours proved to be easily transplantable from egg to egg. But in the adult chicken they rapidly disintegrated. Why should this be the case? In his second paper (198) he attempted to find at what stage in development the power of resistance arose. He came to the conclusion that "the quality which makes the chicken an unsuitable host for the growth of a tissue of a foreign species seemed to develop some time between the eighteenth and twenty-first day." Later, he observed that if a foreign tumour is left in the egg till after the onset of the resistant period, the chick deals with it as if it had suddenly become immune. Refractoriness comes on quite quickly. As late as the morning of the eighteenth day the grafts were found to be in active growth, but late in the evening the mitoses had ceased, and by the following evening degeneration was proceeding rapidly. By the twentieth day the growth was becoming encapsulated by connective tissue. If the tumour was implanted after the eighteenth day, exactly the same changes took place. Murphy remarked, "The period in question is not responsible for any sudden change." As a matter of fact, from the chemical point of view, it is

just at this time that the greatest changes are taking place: we have already had numerous examples of this. But with what particular one of them to correlate a resistance to cancer implantations is an exceedingly difficult problem.

In a later paper (199) Murphy considered the bearing of his results on the theories of Ehrlich and Bashford. According to the former, each sarcoma has a specific food substance which it needs for its growth and maintenance. Temporary survival of the sarcoma in another animal is due to the fact that it carried some of its particular food-substance along with it, and were it not put back in the first animal it would die of inanition, not having the power to manufacture its specific food-substance from the second animal. But Bashford pointed out that since a second graft of the first animal's sarcoma into the second animal dies almost at once—although on the former view it ought to have brought its food substance with it—we must believe that active immunity is possible. Accordingly Murphy argued that if the refractory state in the avian egg is due to the activity of some tissue or organ formerly quiescent, it should be possible to provide the embryo with it by grafting various tissues into the egg from the adult animal. In vitro, it had been found that rat sarcoma growing in chicken plasma was not affected by adult chick connective tissue, kidney or liver, but that when adult chicken spleen was introduced, the growth of the sarcoma received a complete check. Exactly the same results were obtained in vivo. Adult chick spleen, and adult chicken bone marrow—only these two tissues, none others—caused complete degeneration of an implanted tumour, whether they were inoculated before, at the same time, or after it. Nor did it matter whether the inoculations were done on different sides of the egg, thus showing that the effect was due to some mechanism which could act at a distance. The hemopoietic tissues, Murphy concluded, "are capable of supplying a defensive mechanism to the chick embryo, which under ordinary conditions offers no resistance to the growth of foreign species tissue." It is interesting to observe that Apolant (14) had shown previously that in splenectomised animals, only slight immunity to transplantable tumours could be obtained.

With what to correlate these extremely interesting facts on the chemical side is a problem for which it is very difficult to suggest any answers. But its solution should throw a brilliant light on to the whole problem of resistance to new growths.

The energy relations of embryogenesis. The greatest amount of work in this field has been done by Tangl and his collaborators, although perhaps the surest foundations for future work have not been laid by them. In his first paper (268) Tangl defined his aim as the attempt to see how much energy was utilised during development, in what manner the process went on, and what were the sources of it "Die Menge" he said "der während der Entwicklung des Embryos umgewandten chemischen energie, nenne ich Entwicklungsarbeit." It is necessary to say at the outset that in this matter everything depends on definitions, and if we accept this one of Tangl's, then we can accept all his results. But as shall appear later, it is very doubtful whether we can do so, in view of the fact that the embryo is absorbing nourishment all the time in the same way as an adult organism.

Tangl's calorimetric measurements were done by combusting various fractions in the Berthelot-Mahler bomb calorimeter using a pressure of 24 atmospheres of oxygen. The difference between the amount of energy present in the egg at the beginning of development and that present in the embryo at the end, he assumed to have been used in doing the work of development. His first figures were obtained for the eggs of the starling.

	Small calories
Undeveloped egg	3067
Finished embryo	2312
Entwicklungsarbeit =	755

The E_a of the ontogenesis of the starling, accordingly, amounted to 755 calories, a loss of energy of 24.6 per cent, corresponding to a loss of weight of 15.7 per cent. He next estimated the energy-content of the chick at different stages of incubation, and obtained the curves given in figure 33 from which he concluded that the Entwicklungsarbeit of the hen embryo was 1600 calories.

He then went on to define two further expressions, the "Relative Entwicklungsarbeit," and the "Specific Entwicklungsarbeit." The former was the Entwicklungsarbeit expressed in terms of 1 gram of embryo and the latter the same only referred instead to 1 gram of embryo dry weight. These amounts will hereafter be designated as E_a, RE_a and SE_a for greater convenience. For the chick their values worked out at

	calories
RE_a	658
SE_a	3426

Thus 48 per cent of the total energy remained in the yolk at the end of incubation, and whether used in the same proportions thereafter as before hatching, could not be determined. The partition of energy in the developed chick was interesting, but difficult to interpret. Probably it was an index of fat stores.

	PERCENTAGE OF ENERGY-CONTENT OF WHOLE EMBRYO	CALORIES
Muscles	28.3	6687
Central nervous system	3.1	6007
Abdominal contents	17.6	5950
Skin	21.4	5537
Bones	22.4	4907
Remainder	7.2	5647

Tangl devoted his second paper (269) to the question whether a lower animal would need more or less energy for development than a higher one. To gain some light on this subject he examined the growth of bacteria, and concluded that the process of their growth could not be directly compared with embryogenesis. Farkas (76) then published a study on the same lines of the egg of the silkworm, Bombyx mori, and obtained the following values for Tangl's expressions.

RE_a 882
SE_a 3125

This was followed by a paper on marine eggs (273) by Tangl and Farkas together. They chose those of the trout, and were extremely astonished to observe a synthesis of fat to the extent of 37 per cent, a surprise which Gortner was later to share. The E_a worked out at 346 calories and the energy-utilisation, which had been 18 per cent for the hen and 24 per cent for Bombyx, proved here to be as low as 3.5 per cent. The ratio of water lost to dry substance lost was the same for the hen as for the trout but differed considerably for the silk work. The RE_a and the SE_a were not calculable for the trout because the weights of the embryos could not accurately be determined, but it would certainly have been less than the value for the hen because the Chick's per cent energy loss had been so much the greater. As explanation they suggested that perhaps urea and uric acid might be energy-sources; a theory subsequently disproved by Gortner. They looked for glucose and glycogen but could find no trace of either, and finally relied on their suggestion of urea, for reasons derived from the specific energy-content of the eggs.

The next paper was by Tangl and von Mituch (274). They stressed the importance of having all hens on a standard diet and of knowing exactly the time of laying, and they brought forward evidence in confirmation of Krogh and others to show that no volatile nitrogenous compounds were lost by the egg during development. They made further an interesting calculation. Liebermann (165) had shown that the fat of the egg contained 71.67 per cent carbon, and the amount used per egg they had found to amount to 2.11 grams on an average. Hasselbalch (112) had found the total amount of CO_2 produced to be 5.939 grams, i.e., 1.62 grams of carbon. Therefore if all this was the result of fat combustion, 2.26 grams of fat would have to have been used, a figure agreeing fairly well with that of Liebermann and another, 2.76, obtained by the writers themselves. Fat was certainly the preponderating source of energy in the developing avian egg. In this paper the RE_a and SE_a were redetermined, and worked out at 805 and 3600 calories respectively, thus agreeing better with the silkworm figures. The total E_a was also redetermined and proved to be 22,000 calories instead of 16,000—a fact of great importance, as will be seen below.

In the sixth paper of the series (271), Tangl elaborated some further distinctions and laid down their definition. He distinguished between "Erhaltungsarbeit" and the "Arbeit für bildung von lebenden substanz" by saying that out of the total energy at the command of the organism in its development, some—a small part—was used to maintain its state as a physical system (cf. Shearer (256) and Terroine and Wurmser (276)) but by far the greater part was used for the building of living matter. This latter fraction he divided further into "Neubildungsarbeit" and "Wachstumsarbeit." Roughly corresponding to Differentiation and Growth respectively, these terms were to stand for the production of organs, the laying down of morphological and chemical structure, the architectural planning, on the one hand; and on the other hand, the actual increase in size of individual cells and the body as a whole. Not content with definitions, however, he proceeded to try to estimate the apportionment of the Entwicklungsarbeit between these two types of growth. The metamorphosis of insects, he thought, would help to solve the problem. In the metamorphosis of an insect, according to Tangl, the weight loss was so slight as to be negligible, there would be no Wachstumsarbeit, and all that changed would be the form and spatial arrangement of the cells making up their various organs. The metamorphosis of an insect could be considered a case of pure Neubildungsarbeit. In criticism of this view, it is obvious at first sight that

the larva respires feebly and cannot therefore be considered as losing no weight, and secondly, Tangl omitted entirely from his plans the Erhaltungsarbeit, or Energy of maintenance. Moreover, how far insect metamorphosis can be compared with embryonic growth is a question which Tangl never satisfactorily answered. In the metamorphosis process, there is not only the laying down of new structure to be taken into consideration, but there is also the histolysis of the old arrangements. Why should there not be a Histolysearbeit? Moreover, the insect contains a "fat body," which, according to Folsom (84), can be considered as the equivalent of a yolk, and which disappears in metamorphosis as a rule.

Tangl chose for his work the eggs of Ophyra cadaverina, a corpse-fly. It was at once very interesting that the RE_a worked out at 523 calories, and the SE_a at 1566. This showed that the partition between Neubildungsarbeit and Wachstumsarbeit was going to be about 50 per cent. In his following paper the whole matter is set forth in the following table.

	E_a	RE_a or RN_a	SE_a or SN_a
Embryonic development			
	grams—calories	*grams—calories*	*grams—calories*
Hen (Tangl, 251)	23,000	*810*	*3800*
Bombyx mori (Farkas, 76)	408	*880*	*3100*
Fundulus (Glaser, 93)	723		*3280*
Metamorphosis			
Ophyra cadaverina			
(1) Larva, pupa	4340	470	1200
(2) Pupa, imago	3820	520	1600
Total larva, imago	8160	*990*	*2800*
Calliphora vomitoria			
(1) Weinland	275		
(2) Pupa, imago	2430	400	1500
Bombyx mori			
(1) Larva, pupa	4160	320	1400
(2) Pupa, imago	3790	480	2000
Total larva, imago	7950	*800*	*3400*

The conclusions from this are quite definite. In the first place it is clear that the SE_a of the chick and of the silkworm are to all intents and purposes identical. Tangl was therefore justified in saying that "die

specifischeentwicklungsarbeit der Tierischen Organismsn keiner Funktion ihrer phylogenetischen-Stellung und Organisationstufe ist." The close correspondence between the RE_a and SE_a of different insects is striking, and would seem to indicate that the metamorphosis-process is fundamentally alike in all of them.

But, the premises of the work once granted, more significant still is the observation that the RE_a and SE_a of metamorphosis are in all cases only half that of complete embryonic development. Tangl maintained that the direct comparison of insects with the chick was fully justified because the RE_a and SE_a of the chick, the silkworm and the Fundulus corresponded so strikingly. The average figures for metamorphosis were RE_a 437 and SE_a 1550 calories; those for complete development *when halved* worked out at RE_a 422 and SE_a 1720 calories. Tangl concluded from all these data first, that the RE_a and SE_a were not functions of taxonomic position, but were of universal significance, and second, that the energy of growth was equally divided between Differentiation and Growth—simple.

Now if this were indisputable and not open to damaging criticism, it would be a result of enormous importance. But as a matter of fact, as Dr. H. Murray points out in an unpublished paper, it is difficult to accept Tangl's most fundamental conception. To take the simplest case, the energy contained in the egg at the end of development is less than that contained in the egg at the beginning; a certain amount has been lost. There is no doubt about that, it is an experimental fact, but to call that energy the Entwicklungsarbeit, as Tangl does, it very misleading, for to do so assumes that all the energy lost has been utilised for development proper, apart from the maintenance of life. The trouble is that Tangl never allows for the fact that all the time the embryo is developing it is also eating, and in so far as it is doing the latter it is behaving in just the same way as an adult organism. The only real sense, surely, in which the term Entwicklungsarbeit can be used is to denote that portion of energy, if any, retained by the organism in the passage of fuel material into CO_2. In other words, if the amount of heat lost by the egg does not agree with the amount of heat it should have lost, as calculated from the output of CO_2, then it must have retained a fraction for Erhaltungsarbeit, Neubildungsarbeit, Wachstumsarbeit or some other use. If there is any "cost of production" of embryo from yolk and albumen, then direct and indirect calorimetry must give different results. It was in this way that Bohr and Hasselbalch argued, when they determined the heat-evolution and CO_2 pro-

duction of the same egg at different periods of incubation. Their curve for heat in calories per hour has already been referred to (see fig. 6) but they also showed that between the eighth and nineteenth days the calculated and observed curves for heat-production went absolutely together. In other words, there was no retention of heat by the embryo, and therefore no true E_a.

A calculation which shows this for the whole period is easy to make, though it is not exceedingly accurate, owing to the high respiratory quotient of the first five days and complications, due, as Dr. H. Murray ingeniously suggests, to shell carbonates during the last week. The total number of calories actually produced during incubation and measured externally by Bohr and Hasselbalch is 22,000. That lost by the egg measured by combusting the egg, Tangl gave first as 16,000, but afterwards assessed at 22,900, thus coming into line with Bohr and Hasselbalch. Now the total amount of CO_2 expired is 3060 cc. and since this comes principally from fat, calculation gives as the calorific value of this amount 21,430 calories. Therefore no heat is retained and no energy used in cost of production.

Of course, if this is really the case, Tangl's figures must be interpreted anew. The remarkable numerical agreement between the RE_a and SE_a of embryos of different species must mean that the ratio of food-supply to embryo follows a similar curve in all species of eggs, and that their resting metabolism is the same. In metamorphosis the food supply is always much less, and hence Tangl's lower figures. In themselves these conclusions are very interesting but they are entirely different from those that Tangl drew.

We are accordingly driven to believe, then, that one calorie value of albumen and yolk can transform itself into one calorie value of feathers, muscles, blood and brain, without expenditure of energy. It will however, be safer to say for the present, that whether or not there is any true Etwicklungsarbeit, its value, at any rate, must be exceedingly small.

CONCLUSION

This paper has now reviewed, it is hoped, all the work which has been done on the metabolism of the developing egg—the biochemistry of embryology. On more than one occasion it has been noted how difficult correlation is with the data of morphological and histological embryology; a state of affairs largely due to the insufficiently chemical outlook of the earlier embryologists and the lack of interest in the subject on the

part of biochemists. One possible correlation of a general type may possibly, however, be indicated.

In studying the hen's egg, no one can fail to be impressed by the way in which the chemical aspects of events is quiescent till after the second week. It seems clear, on the whole, that this is a real phenomenon, and is not due merely to the coarseness of our estimation procedures. Can it be correlated with any of the data of embryology? In a recent review Huxley (136) says

> According to our present knowledge, three fundamentally different processes, essentially consecutive, though here and there overlapping, are to be distinguished in Amphibian development up to hatching. (1) Division and re-arrangement of pre-existing material and structure; (2) Non-functional differentiation, including (a) Determination and (b) Differentiation of the primordia of organs, and (3) Functional differentiation.

Now is it possible that these three periods may apply also to the avian egg? One pictures the gradual elaboration of structure up to the beginning of the third week, followed then by a burst of chemical activity, consequent upon the assumption of function by the organs already formed. The time of appearance of such functions as kidney secretion fits in with this hypothesis. In it is involved the view that in the first fortnight chemical change is limited to those compounds which are required principally for structural purposes; while towards the end of incubation, the opening up of functional activities causes marked and profound chemical changes of other kinds. The analogy is that of a theatre before a performance of some play or opera. The structure is there, the scenery is ready, the seats are waiting for the audience; and the biochemical transformations which are the index of the beginnings of function correspond to the lighting up of the threatre and the gradual arrival of the spectators. At any rate, this suggestion is quite speculative and there may well be other ways of interpreting the experimental evidence.

Some repetition in this review has been unavoidable. The subject is like a map of a street-car system; there are great complexities and any one point may have connection with three or four others. And while a two-dimensional map of the processes occurring in the developing egg would be difficult enough to prepare, a description of them in one dimension—length—is even more difficult.

The amazing phenomena of embryogenesis have exercised the attention of great numbers of biologists, but since the advent of exact chemical methods, the application of these to embryology has proceeded

slowly; and this is unfortunate, for besides the necessity of the biochemical outlook for a full understanding of embryological processes, the egg is the place par excellence for the study of precursor problems. At any rate, the field lies clear now for chemical methods, and we must look chiefly to them in the future for light on the problem of embryogenesis. If biochemists devote more attention to embryology in the future than they have in the past, a reviewer twenty years hence will probably not be moved to end his paper with that remark of William Harvey's: "Neither the Schools of Physicians, nor Aristotle's discerning brain, have yet disclosed how the hen and its seed doth mint and coin the Chicken out of the Egg."

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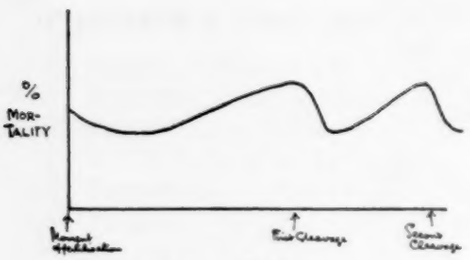


DIAGRAM. I. (Lyon)

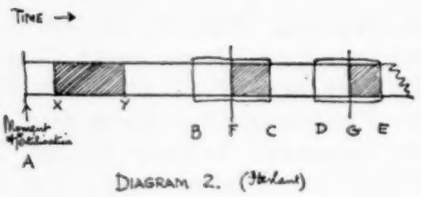


DIAGRAM 2. (Harland)

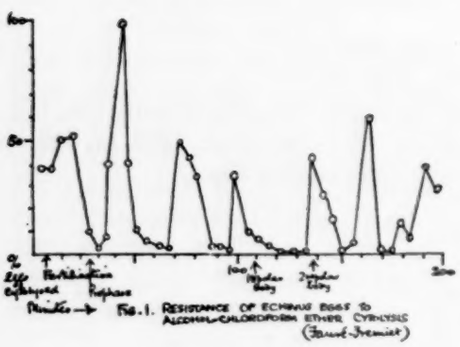


FIG. 1. RESISTANCE OF ECHINUS EGGS TO ALCOHOL-CHLOROFORM ETHER CYANOLYSIS (Faure-Fremiet)

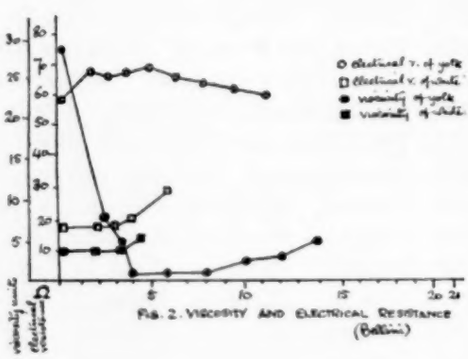


FIG. 2. VISCOSITY AND ELECTRICAL RESISTANCE (Bellini)

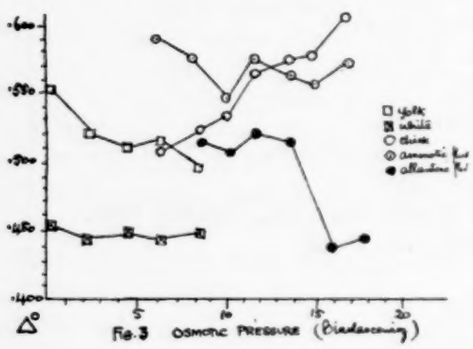


FIG. 3 OSMOTIC PRESSURE (Bialaszewicz)

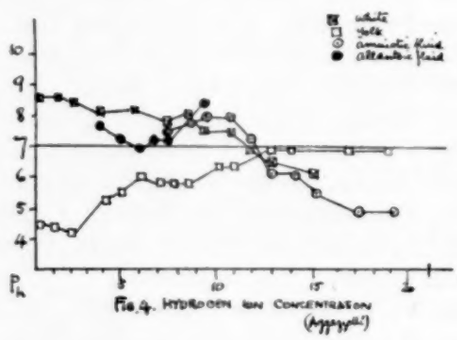


FIG. 4. HYDROGEN ION CONCENTRATION (Aggazzotti)

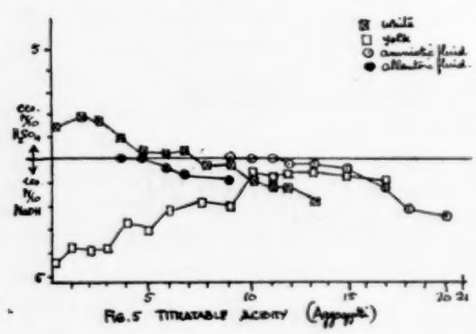


FIG. 5 TITRATABLE ACIDITY (Aggazzotti)

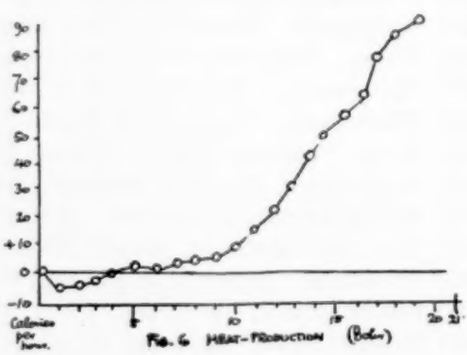


FIG. 6 HEAT-PRODUCTION (Bohn)

FIG. 1

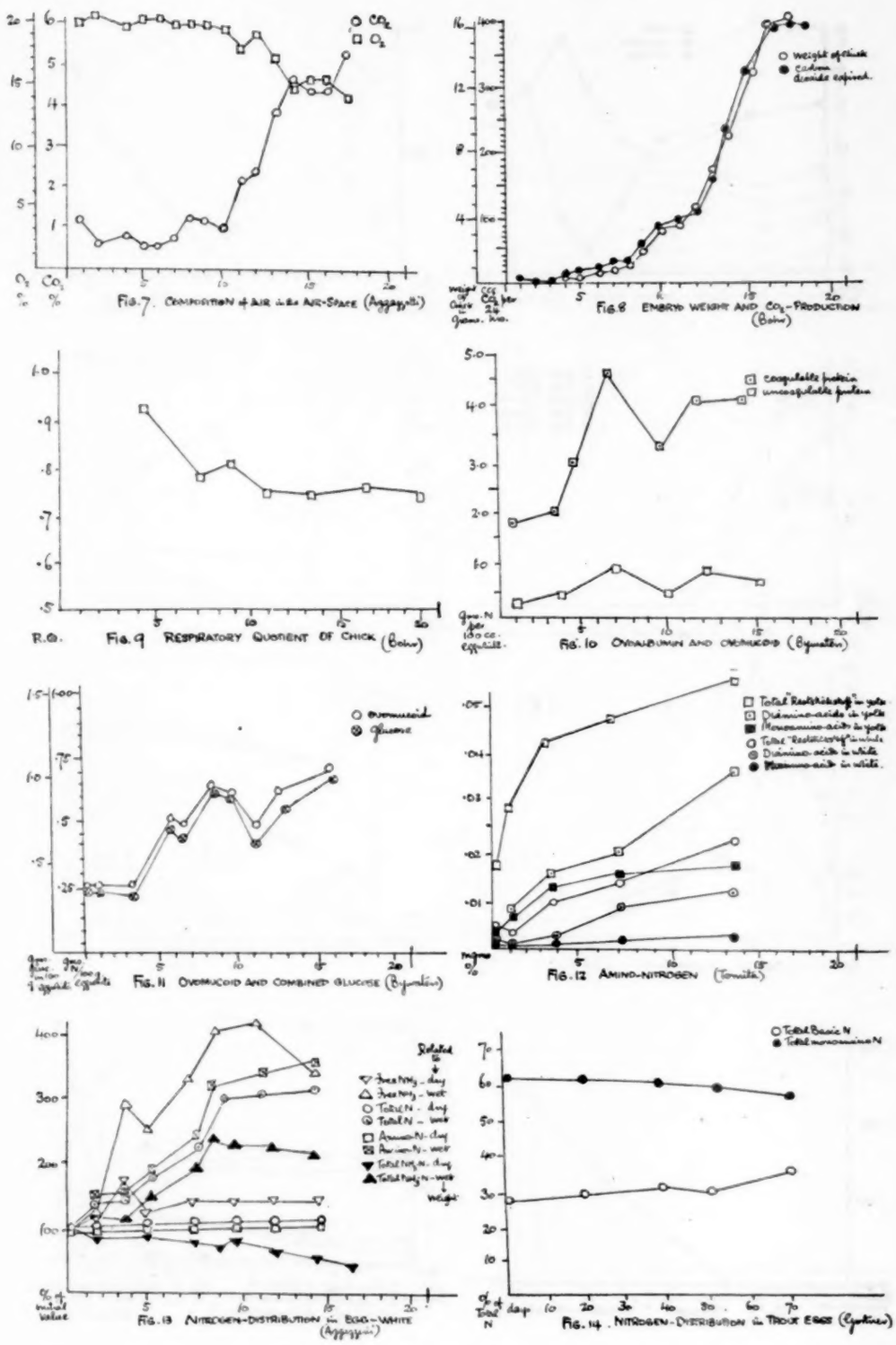


Fig. 7. Composition of Air in Air-Space (Aggazzotti)
Fig. 8 Embryo Weight and CO_2-Production (Bohr)
Fig. 9 Respiratory Quotient of Chick (Bohr)
Fig. 10 Ovalbumin and Ovomucoid (Bywaters)
Fig. 11 Ovomucoid and Combined Glucose (Bywaters)
Fig. 12 Amino-Nitrogen (Tomita)
Fig. 13 Nitrogen-Distribution in Egg-White (Aggazzotti)
Fig. 14. Nitrogen-Distribution in Trout Eggs (Gortner)

FIG. 2

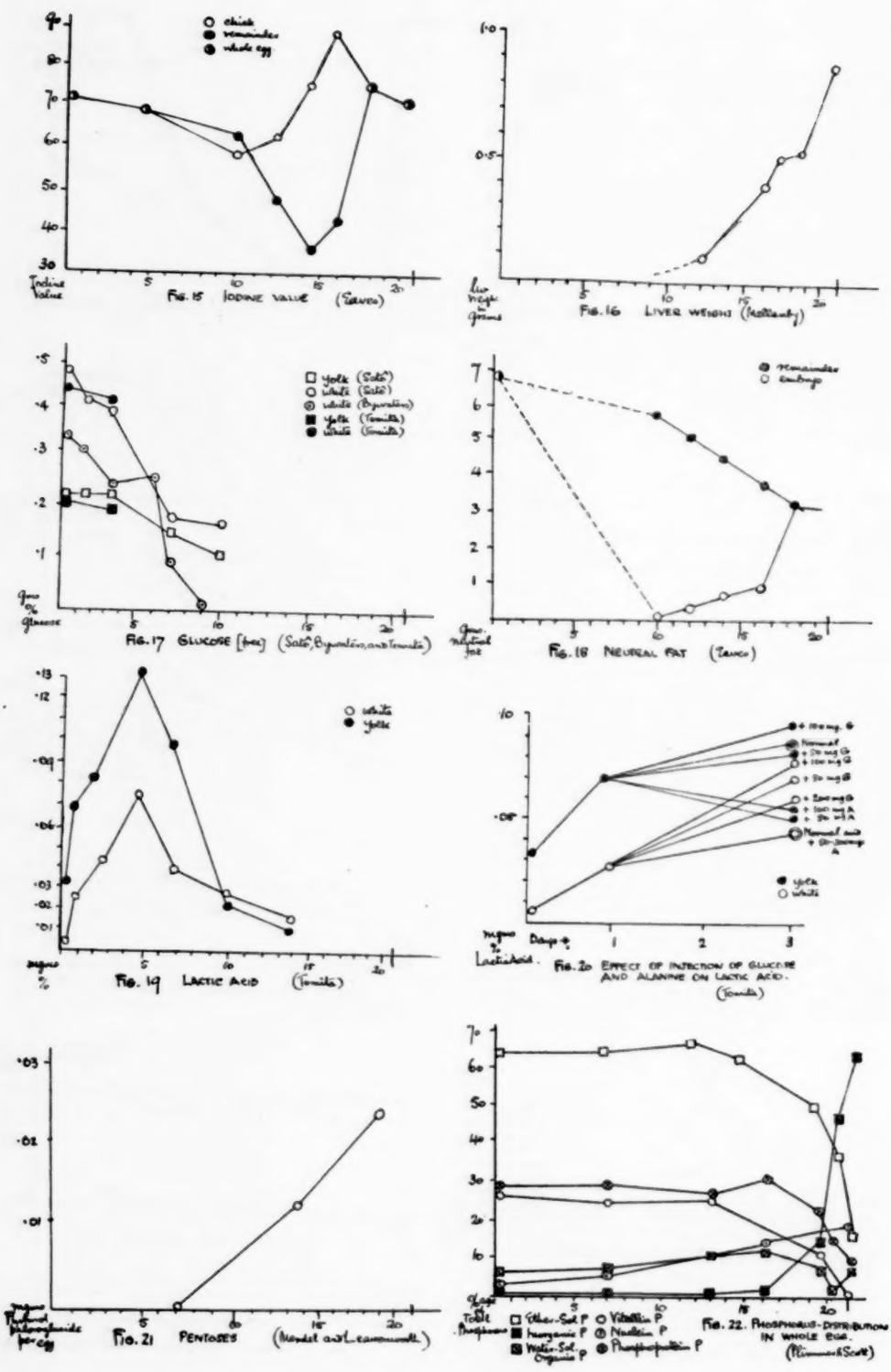


Fig. 15 Iodine value (Tsuboi)
Fig. 16 Liver weight (Kellenberger)
Fig. 17 Glucose [free] (Saló, Byanlén, and Tomita)
Fig. 18 Neutral fat (Tsuboi)
Fig. 19 Lactic acid (Tomita)
Fig. 20 Effect of injection of glucose and alanine on lactic acid. (Tomita)
Fig. 21 Pentoses (Mendel and Leavenworth)
Fig. 22. Phosphorus-distribution in whole egg. (Plimmer & Scott)

Fig. 3

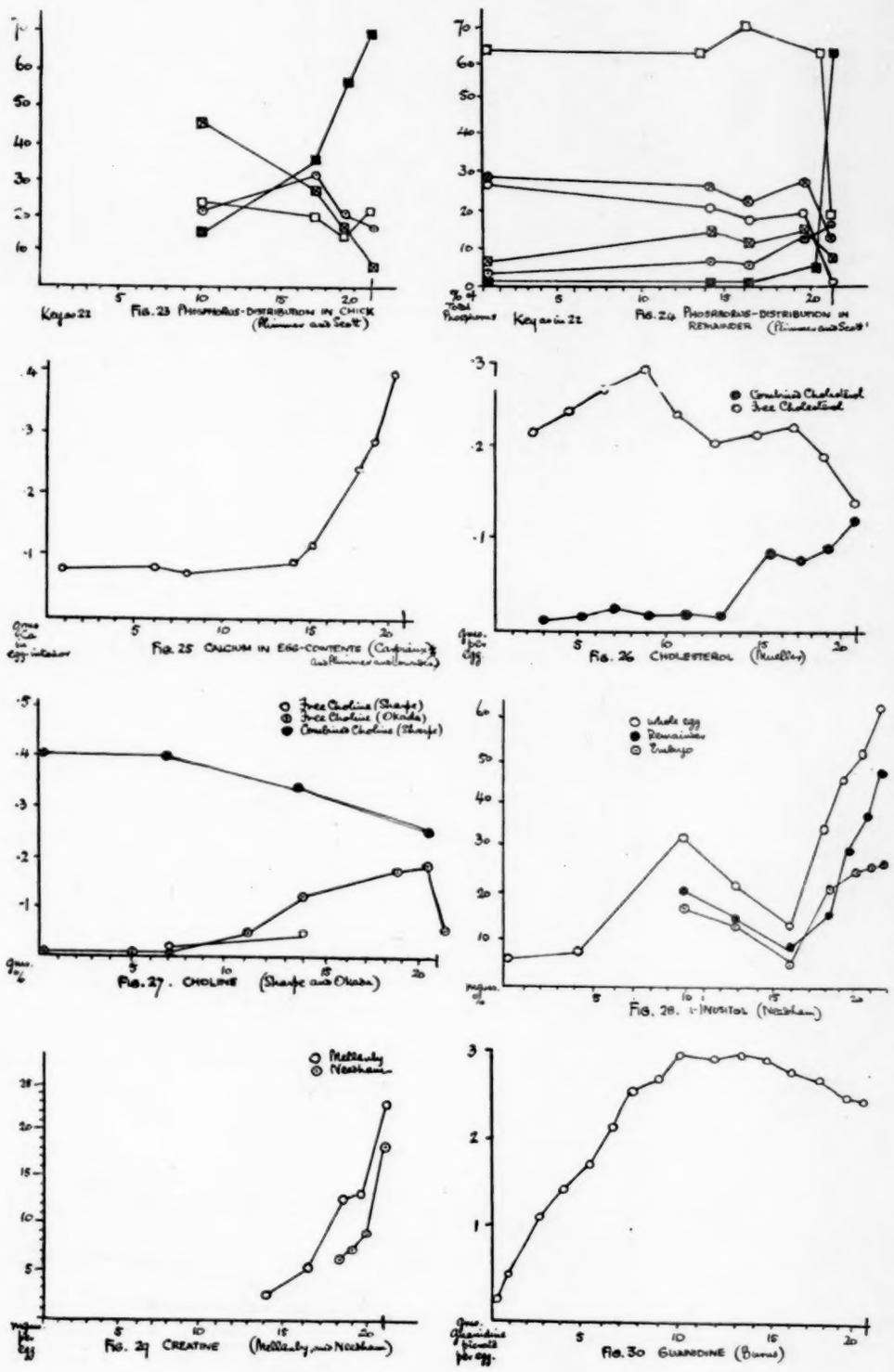

Keys in 22
Fig. 23 Phosphorus-distribution in chick (Rimmer and Scott)
Keys in 22
Fig. 24 Phosphorus-distribution in remainder (Rimmer and Scott)
Fig. 25 Calcium in egg-contents (Cruickshank and Rimmer and Lowndes)
Combined Cholesterol
Free Cholesterol
Fig. 26 Cholesterol (Mueller)
Free Choline (Sharpe)
Free Choline (Okada)
Combined Choline (Sharpe)
Fig. 27. Choline (Sharpe and Okada)
whole egg
Remainder
Embryo
Fig. 28. i-Inositol (Needham)
Mellanby
Needham
Fig. 29 Creatine (Mellanby and Needham)
Fig. 30 Guanidine (Burris)

Fig. 4

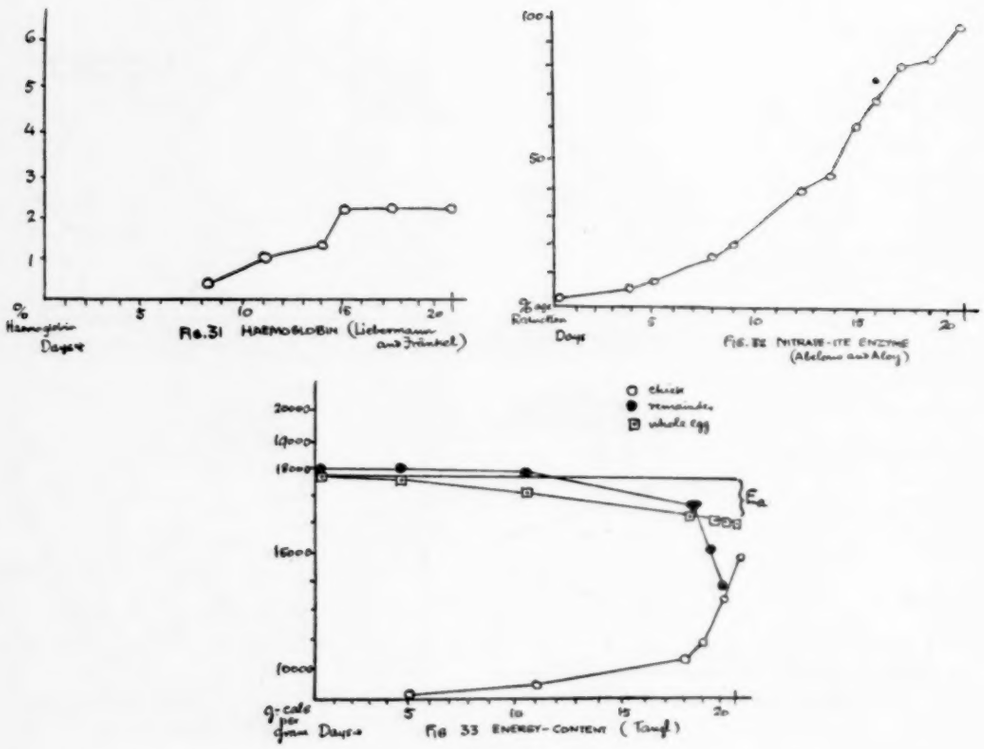

6
5
4
3
2
1
% Haemoglobin
Days
5
10
15
20
Fig. 31 HAEMOGLOBIN (Liebermann and Fränkel)
100
50
% age Reduction
Days
5
10
15
20
Fig. 32 NITRATE-ITE ENZYME (Abelous and Aloy)
chicken
remainder
whole egg
20000
19000
18000
17000
16000
15000
g-cals per gram
Days
5
10
15
20
Fig 33 ENERGY-CONTENT (Terroine)

FIG. 5

THE PHARMACOLOGY OF ARSPHENAMINE (SALVARSAN) AND RELATED ARSENICALS

CARL VOEGTLIN

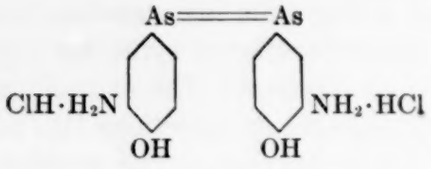
Hygienic Laboratory, U. S. Public Health Service

Ehrlich's epoch-making discovery of arsphenamine, some 15 years ago, was the culminating result of a laborious investigation (more or less empirical in nature) aiming at the elaboration of synthetic chemicals for the specific treatment of infectious diseases. The marvelous effect of this drug on various experimental spirochaete infections (19) justified its immediate introduction into human therapy. The striking early results obtained in the treatment of syphilis, relapsing fever and yaws seemed to confirm the results of animal experimentation. Very soon, however, reports began to appear of toxic reactions occurring in a certain percentage of treated patients and after a short trial it was found that Ehrlich's expectation of producing a cure by a large single injection (Therapia sterilisans magna) was not always fulfilled in some of these diseases. In retrospect it is now possible to understand some of the reasons for the toxic reactions and the insufficient sterilizing power. It is important to realize that very little was known concerning the pharmacological action of arsphenamine at the time of its introduction into therapeutics beyond the fact that it had powerful spirochaeticidal properties, and caused the rapid destruction of the parasites and the healing of specific lesions in doses which were considerably below the fatal dose. The need for a thorough analysis of the pharmacological action of arsphenamine, a need which no one appreciated more than Ehrlich himself,[1] therefore became an urgent problem. In the case of drugs of the arsphenamine type the pharmacologic action deals of course with the study of the action of the remedy, 1, on the higher animals; and 2, on the specific organisms without and within the mammalian host. The following pages will be devoted to a critical summary of this complex subject. At the outset it should be stated that much valuable informa-

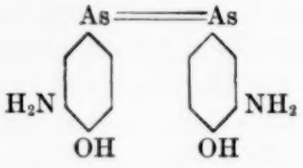
[1] In all his publications on chemotherapy Ehrlich emphasizes the extreme importance of the study of the mechanism of drug action and his chemoreceptor theory is a striking example of his efforts along this line.

tion has accumulated since the year 1910, but as Dale (12) has shown in a recent masterly review of the whole subject of chemotherapy, we are far from the goal, i.e., a complete understanding of the chemotherapeutic process.

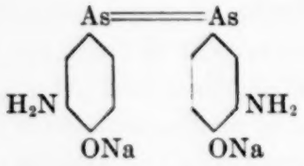
Chemical constitution, chemical and physical properties. There is no class of drugs in which the intimate relation between the chemical and physical properties on the one hand and physiological effect on the other is more in evidence than in the case of the organic arsenicals. The chemical and physical properties, of course, are inherent in the chemical constitution, which as far as known, is given below:



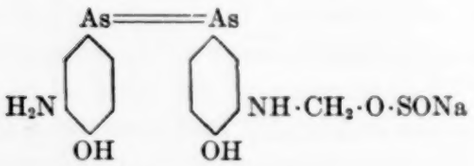
Arsphenamine



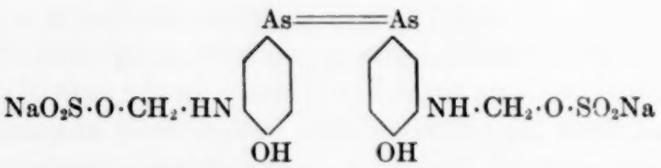
Arsphenamine base



Sodium salt of Arsphenamine



Neoarsphenamine



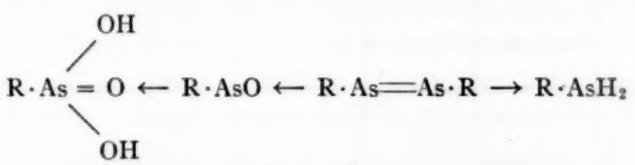
Sulpharsphenamine

Coördination products of arsphenamine and heavy metal salts have also been found of some therapeutic use, especially silver arsphenamine and neosilver arsphenamine. The chemical constitution of these metal derivatives is not definitely known at present. Ehrlich and Karrer (20) are of the opinion that the metal is attached to the secondary valen-

cies of the arsenic; Binz, Bauer and Hallstein (7) maintain that the metal is attached to the amino groups, but recent results of Raiziss and Gavron (57) seem to indicate that we are dealing with an intimate mixture of arsenicals with colloidal metal. Many other derivatives of arsphenamine have been introduced but their practical importance is but slight and theoretically they are not of any particular interest.

None of these drugs, as used in practice, is a chemically pure compound. Considerable work has been done on the chemical nature of the impurities and their relation to the method of preparation and the toxicity of the resulting products. There is no question that relatively slight variations in the conditions of synthesis may have a profound influence on the physiological action, a fact which makes it necessary to test these drugs biologically prior to their use in medicine.

Arsphenamine and its derivatives are trivalent arsenicals. Reduction of arsphenamine leads to the corresponding arsine, partial oxidation of arsphenamine to the trivalent arsenious oxide, which in turn can be further oxidized to the corresponding pentavalent arsenical.[2] This relationship may be expressed as follows:



where R represents the substituted benzene ring. The biological significance of this series of compounds will be discussed later on.

It was first clearly shown by Bauer (6) and confirmed and amplified by Raiziss and Gavron (57), that arsphenamine behaves like a semicolloid. The drug dializes very slowly through parchment and somewhat more rapidly through collodium membranes. Experiments on the diffusion through gelatin gel furthermore brought out the fact that the salts of the base are easily hydrolyzed. Highly concentrated aqueous solutions of arsphenamine on standing have a tendency to gel and the drug can be "salted out" from its concentrated solution by the addition of NaCl. Arsphenamine base, on account of its basic amino groups and acidic phenolic groups, may be considered as a complex ampholyte, whose conduct is profoundly influenced by the hydrogen ion concentration of the solution. In this sense, arsphenamine has great similarities

[2] The chemistry of these arsenicals is discussed in the recent monograph by Raiziss and Gavron, *Organic Arsenical Compounds*, 1923.

with proteins. The acid-base equilibria of arsphenamine have been studied recently by Elvove and Clark (21). A typical titration curve is given in figure 1. The dihydrochloride has a pH of about 2.36, the monohydrochloride 3, and the disodium salt 11.32. From the physiological standpoint it is significant that the so-called isoelectric point of the ampholyte lies in the neighborhood of the reaction of the blood, hence only a very small degree of ionization can occur at blood pH. At

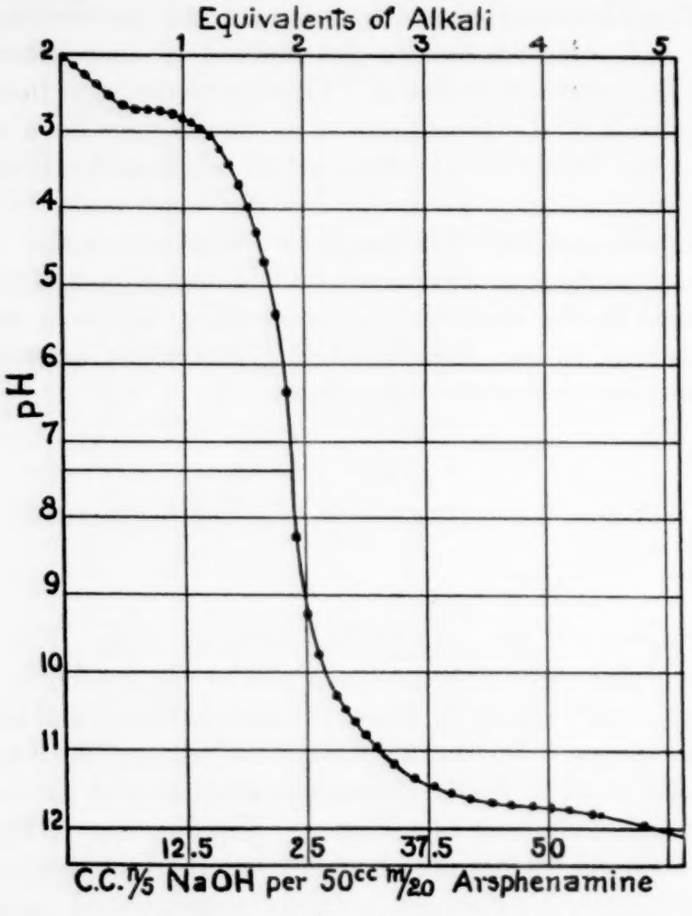


FIG. 1

this pH the base is thrown out of solution as a gelatinous mass. The figures given above show that the hydrochloride is extremely acid and the disodium salt very alkaline. That intravenous injections of such solutions are inimical to animals and man will be shown later on. The semicolloid character of arsphenamine reveals itself also in the viscosity of aqueous solutions, which has been studied quantitatively by Klemensiewicz (39) and Voegtlin, Johnson and Dyer (79). The viscosity of the

dihydrochloride increases very rapidly with the concentration and on standing of such solutions, whereas dilute solutions become less viscous on standing. The viscosity of the sodium salt on the other hand is not much higher than that of water, indicating a high degree of dispersion.

Arsphenamine, as all other arseno compounds, is readily oxidized under certain conditions, this being accomplished by the splitting of the As═══As linkage and the addition of oxygen to the arsenic. As shown by Voegtlin and Smith (81) this oxidation is largely determined by the pH of the solution, the hydrochloride and the free base being fairly stable, whereas the alkaline solution is rapidly oxidized, the rate of oxidation increasing with an increase in pH.

The striking effect of chemical substitution in the amino group of arsphenamine on the physical and chemical properties of the resulting compounds is illustrated in the case of neoarsphenamine, sulpharsphenamine and the diglucanol arsphenamine (condensation product of arsphenamine and glucose (Aubry and Dormoy, (4)). This substitution, in every case, leads to a greater solubility over a wider range of pH and as far as is known, to greater dispersion (Bauer, Raiziss and Gavron). All three substitution products are easily soluble in water at blood pH, and appear to diffuse more easily than arsphenamine. Neoarsphenamine is exceedingly unstable in solution in contact with oxygen and oxidizes very rapidly. Sulpharsphenamine, as shown by Voegtlin and Johnson (77), is acid in reaction and extremely resistant to oxidation by air, a matter of practical importance. These workers have also described a simple differential test for neoarsphenamine and sulpharsphenamine which is based on the fact that neoarsphenamine readily reduces a solution of indigo-carmine, indicating oxidation of the side chain, whereas the test is negative with sulpharsphenamine. The digluconyl arsphenamine is practically neutral in reaction, slightly levorotary and is readily hydrolyzed under certain conditions with the reformation of glucose and arsphenamine.

To sum up, it appears very clearly from this cursory presentation that the chemical and physical properties of arsphenamine and its substitution products are determined by their peculiar chemical constitution. The subsequent discussion will attempt to correlate as far as this is possible the physicochemical and physiological properties. It should be emphasized at this point that for a better understanding of their pharmacological action further work on the physicochemical behavior of these compounds and particularly of those of the corresponding oxide and pentavalent arsenicals is greatly needed.

Pharmacological action in mammals. In recent years evidence has accumulated clearly indicating that the physiological action of arsphenamine (and perhaps to a lesser extent of its substitution products) can be separated into 1, reactions attributable to the physical properties; and 2, other reactions which are due to the chemical properties of the drug. It is perhaps permissible to speak of a "physical" and a "chemical" toxicity of arsphenamine. The former is related to the physicochemical, particularly the colloidal, properties and the latter is due to the chemical reactions occurring between the arsenic of the molecule and certain tissue constituents.

The so-called *physical toxicity* manifests itself especially after intravenous injections, whereas it is very slight or entirely absent after subcutaneous or intramuscular injections. The rather sudden toxic reactions observed clinically and generally spoken of as "nitritoid crises" or "anaphylactoid" reactions occur soon after injection and may lead to sudden death. An experimental analysis of these reactions in animals has produced the following facts. Severe disturbances of the cardiovascular apparatus are noted after a rapid injection of arsphenamine into a leg vein (5), leading to a gradual severe fall in arterial blood pressure. Jackson and Smith (34), Smith (68), Jackson and Raap (33), submitted this action to further analysis and found that this shock-like condition is preceded by a great rise in pulmonary blood pressure and dilatation of the right side of the heart, causing secondarily a rise in pressure in the inferior vena cava and a great fall in pressure in the carotid artery. If on the other hand the drug, instead of being injected into a femoral vein, is infused into the portal vein or the femoral artery only a very insignificant disturbance of the circulation is produced. As the bronchioles do not react by constriction the circulatory disturbances following intravenous injections probably have their primary origin in an increased resistance in the pulmonary circulation. The cause of this increased resistance is very probably due to embolism or in the case of solutions of the disodium salt partly due to the constricting effect of the excess alkali (pH 11) on the pulmonary blood vessels. The formation of precipitates by adding arsphenamine solutions to blood plasma or serum can easily be demonstrated *in vitro* and by examination of pulmonary blood if arsphenamine as the hydrochloride is injected into an animal (35), (14), (68). Alkaline solutions of arsphenamine produce less precipitate with blood than solutions of the hydrochloride, a fact which is probably explained by the dispersing effect of the excess alkali of the disodium salt. It was found by

chemical analysis in the writer's laboratory (unpublished experiments of C. N. Meyers) that these blood precipitates consist of protein and arsphenamine.

Some recent interesting observations by Oliver and Douglas (51), Oliver and Yamada (53) have thrown new light on this subject. In confirmation of Karsner and Hanzlik (37) they found that arsphenamine *in vitro* is absorbed by red blood corpuscles and agglutination occurs in the presence of electrolytes. The agglutinating action was also observed after intravenous injections into rabbits and with moderate doses reached such proportions that multiple emboli were found in many of the organs, especially in the lung. The whole capillary system of this organ may be seen filled with these masses of agglutinated cells. Attention is called to the close analogy of the symptoms of fat embolism, and those following arsphenamine injections. Both conditions are explained on a purely physical basis.

In view of the fact that arsphenamine is a semicolloid and insoluble at blood pH it is not at all surprising that the drug should cause agglutination of red cells and precipitates of the base in combination with blood proteins. The fact that injections into the portal circulation or the femoral artery are so well tolerated is explained by local filtration, phagocytosis, or both, of the precipitates and agglutinated red cells. Later (31) it was found that the rise in pulmonary pressure is gradually reduced in the course of time indicating a gradual removal of the emboli. The extent and the suddenness of the rise in pulmonary blood pressure increases with the concentration and rate of injection of the solution. Similar observations were made on the influence of rate of injection and concentration on the fatal dose in rats (Roth and Lake). The present clinical practice of using dilute solutions of arsphenamine injected very slowly is therefore well founded on experimental evidence. It is of interest to note that neoarsphenamine injected under the same conditions as arsphenamine does not cause any appreciable circulatory disturbances, which may be explained by the fact that neoarsphenamine in solution is more dispersed, only slightly alkaline, and readily soluble in the range of physiological pH. Neoarsphenamine can therefore be injected in much more concentrated solutions and this may also be done in the case of sulpharsphenamine, a substance which with exception of its acid reaction has physical properties very similar to those of neoarsphenamine.

Hunt (30) observed that the toxicity of an alkaline solution of arsphenamine under conditions which preclude oxidation decreases on

short warming or standing of the solution. This fact may also be taken as evidence of the existence of a physical factor in the toxic action. Finally, Voegtlin, Johnson and Dyer (79) were able to correlate quantitatively a purely physical property, i.e., the viscosity with the toxicity of arsphenamine solutions (both acid and alkaline). Solutions of the hydrochloride have a high viscosity and high toxicity, both properties decreasing simultaneously on standing of the solution, whereas solutions of the sodium salt have a much lower viscosity and are much less toxic. These findings serve as an experimental explanation of the sudden fatalities which have sometimes occurred in the clinic, when by mistake the drug was injected without previous alkalinization.

A successful attempt has been made to reduce the physical toxicity of arsphenamine solutions by the addition of protective colloids. Oliver and Yamada (53) found that the relative effectiveness of hydrophilic colloids on the reduction of agglutination of red blood corpuscles by arsphenamine *in vitro* increases in the following series: Starch, egg albumen, gum arabic, gelatine, blood serum. Arsphenamine when mixed with blood *in vitro* or *in vivo* strongly inhibits coagulation, and the fibrinogen becomes incoagulable to both heat and thrombin (51). A similar effect was noted after large doses of neoarsphenamine by Amoyl-Davies and Mellanby (2), whereas therapeutic doses appear to have no such effect. In order to explain this action, it was assumed that these arsenicals enter into combination (physical or chemical) with some of the plasma proteins. This explanation appears to be confirmed by the previous observations of Young (86), who showed that arsphenamine dializes very slowly or not at all from a mixture with blood plasma, indicating some sort of combination of arsphenamine with a plasma constituent. Oliver and Douglas (52) then found that the plasma globulins actually combine with arsphenamine, the degree of this combination being largely determined by the pH, an acid reaction favoring the process and an alkaline reaction inhibiting it. If a large enough dose of the disodium salt of arsphenamine is injected intravenously all of the available fibrinogen combines with arsphenamine and any excess of the drug is bound to the red corpuscles, causing their agglutination. If, however, hydrophilic colloids (gelatine) are added to the arsphenamine solution before injection, these colloids combine with the drug and thus protect the fibrinogen and the red cells. Oliver, Yamada and Kolos (54) have actually proved that the addition of certain hydrophilic colloids (especially gelatine) to an alkaline arsphenamine solution results in a considerable decrease in toxicity, i.e., maximum tolerated dose of arsphenamine 0.09

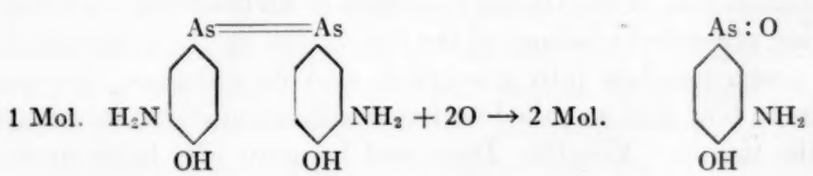
gram per kilogram, of gelatine-arsphenamine 0.14 gram per kilogram. Even very large doses (0.5 gram per kilogram) of gelatine arsphenamine injected intravenously do not produce the characteristic symptoms referable to the physical toxicity of arsphenamine. There is no *intra vitam* agglutination of red cells, no hemolysis, no severe alteration of the circulation and no reduction of the coagulability of the blood.

Attempts have also been made to prevent the physical toxicity of arsphenamine from manifesting itself or to combat its effect by the injection of epinephrin or tyramine (34), on the assumption that the increase in arterial blood pressure produced by these drugs may prevent or remove the obstruction of various capillary fields by emboli. The clinical results obtained by this method have been decidedly encouraging.

Before leaving the discussion of the physical toxicity reference should also be made of the fact that solutions of arsphenamine have a hemolytic action on blood *in vitro* (65), (43), (26). This action evidently depends on the osmotic pressure and the pH of the solutions, being considerable in the cases of the hydrochloride and the disodium salt. The addition of serum inhibits the hemolytic action. Isotonic solutions of neoarsphenamine are not hemolytic. Properly prepared solutions of these drugs do not produce hemolysis *in vivo*.

The so-called "Wasserfehler" or acute reactions observed clinically when old water containing bacteria is used for the preparation of arsphenamine solutions, is probably due to some colloidal reaction occurring between the drug and the foreign protein (85). It has therefore been the practice for years to use freshly distilled water for making the solutions.

The *chemical toxicity* of arsphenamine, and its derivatives has been attributed by Voegtlin and Smith (81) to the directly active modification which is formed in the body by the gradual and partial oxidation of the drug to the trivalent oxide:



For the sake of convenience the latter substance will be designated as "Arsenoxide." This conception is supported by the following facts: *1*, Late appearance of toxic symptoms after an intravenous injec-

tion of arsphenamine under conditions which reduce the physical toxicity to a minimum (alkaline solution, high dilution and slow rate of injection). *2*, On the basis of arsenic content arsphenamine is 7.5 times, and neoarsphenamine 10 times less toxic than arsenoxide. *3*, Alkaline solutions of arsphenamine and neoarsphenamine solutions which have undergone partial oxidation, as a result of standing in contact with air (16), or after short shaking with air (62), (30), (64), are far more toxic (acute symptoms, short survival and lower M L D) than solutions which have not been partially oxidized. *4*, Arsphenamine has very little if any toxicity when tested on the isolated heart (3) and on the nerve muscle preparation of frogs (36). *5*, Arsphenamine *in vitro* is readily oxidized in alkaline solution to the corresponding pentavalent arsenical (18), a reaction which proceeds through the arsenoxide stage: $R{\cdot}As{=}As{\cdot}R \rightarrow R{\cdot}As{:}O \rightarrow R{\cdot}AsO_3H_2$. Further indirect evidence will be presented in the chapter on the parasiticidal action.

These facts may be considered as fairly conclusive evidence of the *intra vitam* arsenoxide production, though the final proof, i.e., the actual isolation of arsenoxide from the tissues after an injection of arsphenamine has not yet been accomplished. This is an extremely difficult task because of the relatively small amounts of arsenoxide present, its great chemical reactivity and the ready oxidation of the substance in the process of isolation.

The mechanism responsible for the *intra vitam* oxidation of arsphenamine and its derivatives to arsenoxide has not been sufficiently elucidated. In the case of neoarsphenamine, this oxidation is probably accomplished by oxygen analogous to the ready oxidation of the drug *in vitro*. Arsphenamine, however, as noted by Voegtlin and Smith (81), is quite resistant to oxidation by air at physiological pH. In some unpublished work (Voegtlin, Johnson and Dyer) it was shown however that in the presence of heavy metal salts and particularly iron, arsphenamine is easily oxidized *in vitro* at pH 7.6. It is therefore very likely that the physiological iron of the tissues functions as an oxidizing catalyst.

Having presented evidence of the conversion by the mammalian body of the arsphenamines into arsenoxide and its anlogues, the question arises as to how this so-called active modification exerts its toxic action upon the tissues. Voegtlin, Dyer and Leonard (74) have shown that arsenoxide appears to react with the SH group of glutathione. This important substance was recently isolated by Hopkins (29) from liver, muscle and yeast, occurs in all tissues with an active metabolism, and is composed of glutaminic acid and cystein. Hopkins and his collabora-

tors have shown that glutathione is concerned in the physiological oxidation-reduction phenomena, functioning as a coenzyme in the transport of hydrogen, thus:

$$\left.\begin{matrix} R\cdot S \\ | \\ R\cdot S \end{matrix}\right\} + 2H \rightleftharpoons 2\ R\cdot SH$$

Oxidized glutathione — Reduced glutathione

Under physiological conditions this equilibrium is well adjusted. Now it is known that arsenoxide and similar trivalent arsenious oxides readily react with H_2S and certain organic sulphydril compounds (thioglycollic acid) with the formation of condensation products:

$$R\cdot As{:}O + \begin{matrix} HS\cdot R \\ HS\cdot R \end{matrix} = R\cdot As\begin{matrix} S\cdot R \\ S\cdot R \end{matrix} + H_2O$$

For this and other reasons (relative mass of glutathione in tissues and toxic dose of arsenoxide) it seemed likely that proof for this hypothetical action of arsenoxide upon the glutathione of the body might be obtained by testing the detoxicating action of various sulphydril compounds on the arsenoxide toxicity. It was found that the time of death of animals injected intravenously with a fatal dose of arsenoxide was greatly delayed by a simultaneous injection of sodium thioglycollate. It was furthermore shown in a paper which is in press,[3] that reduced glutathione completely protects rats against a fatal dose of arsenoxide, whereas cystein is considerably less effective. Furthermore, it was observed that feeding of fairly large amounts of glutaminic acid and cystin (the component amino acids of glutathione) will offer considerable protection against a M L D of arsenoxide injected two or three hours later. In view of the negative results obtained with other amino acids and a great variety of other substances not containing mercaptan sulphur the conclusion may therefore be justified that glutathione in its reduced form is a physiological antidote against arsenoxide and that the action of arsenic upon protoplasm is essentially due to an interference with the normal functioning of glutathione in the oxidation-reduction phenomena of the tissues. The injection of a toxic dose of arsenic leads to cellular asphyxia and hence arsenoxide may be considered as a specific poison for reduced glutathione. Parenthetically, it may be

[3] This paper will appear in the Journal of Pharmacology and Experimental Therapeutics.

stated that these observations illustrate the value of pharmacological studies in the elucidation of certain physiological problems, for at present there is available no better proof of the real physiological importance of glutathione than the observations just described.

The significance of the above theories of the physical and chemical toxicity will now be applied to a discussion of the absorption and the distribution in the body, the excretion and the histological changes produced by the arsphenamines.

From what has been said it is obvious that the physical and chemical properties of the drugs should have a marked influence on the rate of absorption. Arsphenamine in the form of its hydrochloride or sodium salt is highly irritating to tissues, a fact which is partly explained by the physiologically abnormal pH of such solutions and the precipitation of the free base caused by the buffer action of the tissues. The precipitated drug is probably slowly oxidized locally to arsenoxide, which is highly toxic for tissues. Neoarsphenamine on account of its ready oxidation also produces considerable though less marked local irritation and for this reason these two drugs are injected intravenously in the clinic. The same holds true of the silver arsphenamines. Sulpharsphenamine is well tolerated in small doses (children) by subcutaneous injection and in larger doses intramuscularly in adults (69).

If the *rate of absorption* can be judged from the magnitude of the therapeutic effect produced, the rate decreases from the intramuscular to the subcutaneous, rectal and oral administration in the order named. Chemical methods should be used in order to fill out this gap.

Arsphenamine and its derivates are rapidly removed from the blood after their intravenous administration, as shown by curves of the arsenic content of the blood of rats and rabbits (84), (83), and of human blood (41), (22), (23), (24). *The rapid disappearance of the arsphenamines from the circulating blood* is probably very largely due to physicochemical factors such as the previously mentioned combination of the drug with blood and tissue colloids in which process phagocytosis by endothelial cells may also play a significant rôle.

The *distribution of arsphenamine in the body* after intravenous injections has been studied by means of estimations of the total arsenic and by a colorimetric method (41) which indicates the presence of the aromatic amino groups of arsphenamine (Abelin reaction). The former method of course does not furnish any information as to the form in which the arsenic actually occurs in the tissues and the colorimetric method has the defect that it is an indirect method. Table 1 illustrates,

however, the fairly close agreement of the data obtained by total arsenic estimations (83) in rabbit tissues with those obtained by Kolls and Youmans (41) by the colorimetric method applied to the tissues of dogs. The former figures multiplied by three give the approximate weight of arsphenamine and these latter figures are then directly comparable with the data of Kolls and Youmans. These data show that the spleen and liver contain more of the drug, weight for weight, than any other tissue and when the large relative size of the liver is given due consideration, it is evident that this organ forms a regular storehouse for the

TABLE 1

Distribution of arsphenamine in the tissues of the rabbit and dog after a single intravenous injection of 40 and 20 mgm. per kilogram respectively

ORGAN	RABBIT (100 MIN. AFTER INJECTION) MILLIGRAMS OF ARSENIC PER 100 GRAMS FRESH TISSUE	DOG (195 MIN. AFTER INJECTION) MILLIGRAMS OF ARSPHENAMINE PER 100 GRAMS FRESH TISSUE
Spleen	1.68	6.0
Liver	1.61	4.0
Lung	0.39	3.2
Heart	0.28	3.1
Kidney	0.25	3.0
Pancreas	Not determined	2.9
Intestine	0.70 (plus contents)	2.6
Skeletal muscle	0.04	2.5
Brain	0.11	Less than 1.0
Skin	0.18	
Embryo	0.02	
Bile	19.65	
Cerebrospinal fluid	0.72	
Blood	0.52	

drug. It is also of interest to call attention to the fact that Hopkins considers the liver richer in glutathione than any other tissue, and this fact may be partly responsible for the retention of the arsenic by this organ. The high arsenic content of the bile is especially significant and indicates that the liver actively secretes the drug or some derivative thereof. The spleen probably owes its high drug content to its function as a scavenger, catching all altered (agglutinated) red blood corpuscles as well as precipitates. The brain contains a very small amount of arsenic and it is a curious fact that the spinal fluid should show a

slightly higher arsenic content than the blood. Bulmer (10) furthermore called attention to the long retention of arsenic by the femur of rabbits. It is probable that most of this arsenic is retained by the bone marrow. Small traces of arsenic find their way to the placenta and the fetal tissues after intravenous injections of arsphenamine and neoarsphenamine (71), a fact which is of importance in the ante-natal treatment of syphilis.

The *excretion* has been studied very extensively in animals and man (25), (84), (10), (72), (41). The essential facts are as follows: Arsenic appears in the urine within a few hours after an intravenous injection of arsphenamine or neoarsphenamine, the maximum excretion occurring on the day of or the day after injection and small amounts are excreted for a large number of days thereafter. In the case of arsphenamine most of the arsenic is excreted with the feces, this being explained by the high arsenic content of the bile. Animals with a biliary fistula excrete very little arsenic with the feces. A larger percentage of the arsenic of neoarsphenamine appears in the urine and less in the feces than in the case of arsphenamine. There is no relation between the amount of arsenic excreted and the quantity of urine and feces. As would be expected the rate of excretion of arsphenamine after intramuscular injection is considerably lower than after intravenous injection (25). A small amount of arsenic is excreted with the milk for fairly long periods (23), (24). In an interesting study of the excretion of arsenic after serial administration of arsphenamine and neoarsphenamine, Underhill and Davis (72) obtained evidence indicating that these drugs are retained by the body "up to a point at which the tissues are, as it were, saturated with them. When this point has been reached further additions of the arsenical preparations are in large measure quickly eliminated from the body." The evident cumulative action is of very great significance from the therapeutic standpoint.

We know very little about the form in which the arsenic is excreted. It is possible that a small part of arsphenamine is excreted as such in the urine, a view which is supported by the rapid appearance of arsenic and the positive Abelin reaction in the urine after intravenous injections. On the other hand, we have good reason to believe that the larger part of the drug is altered before excretion takes place. The first step in this breaking down of the molecule is the partial oxidation of the As═══As linkage to the arsenoxide form. This latter substance combines with the reduced glutathione of the tissues and is evidently held back tenaciously by the body as indicated by the slow rate of excre-

tion of its arsenic (84). Whether the arsenic is then further oxidized to the pentavalent stage, analogous to the oxidation of the drug *in vitro*, is not known at present, but this is more than probable as Voegtlin and Thompson (84) have shown that all pentavalent organic arsenicals are very rapidly eliminated by the kidney. It is less likely that inorganic arsenic is split off from its linkage to the benzene ring, as it has been shown by Voegtlin and Thompson that this inorganic arsenic is more slowly excreted than arsphenamine itself. The only attempt at the actual isolation of arsenicals from the urine after the injection of arsphenamine was made by Sieburg (66), who used a method yielding untrustworthy results. He evaporated the neutralized urine on the water bath, a procedure which may lead to the splitting off of the arsenic from its organic combination, as was actually shown in the case of solutions of atoxyl. In spite of this defective method he was able to isolate two crystalline substances which on analysis appeared to be amino-hydroxyphenylarsonic acid and hydroxyphenylarsonic acid. There was also found some arsenic and arsenious acid, carboxyl-o-aminophenol, and 2-acetylaminophenol sulphonic acid. It is quite possible that these last mentioned substances were not excreted as such, but rather represent some decomposition products formed in the process of their isolation.

A vast amount of work has been done on the *histological changes* produced by arsphenamine and related arsenicals. These changes, with exception of those due to the physical toxicity (emboli and its results) may be attributed to true arsenic (arsenoxide) effects. The latter vary considerably with the chemical constitution of the drug (55), (42), (87), and the diet of the animals (28). Here again we meet with evidence indicating the greater toxicity of arsenoxide and its derivatives, the active modification as compared with that of the arsenobenzenes and the pentavalent arsenicals.

Hooper, Kolls and Wright (28) produced liver necrosis and jaundice in dogs by large doses of arsphenamine. Histological changes are also found in the kidney, adrenal, lungs, myocardium, blood vessels and spleen. Arsphenamine is more hepatotoxic and less nephrotoxic in rats than equimolecular doses of neoarsphenamine. The dermatitis occurring sometimes clinically after treatment with the arsphenamines has not been observed in animals. It is very likely due to an arsenoxide action on the skin. Moore and Keidel (49) have also called attention to some rare cases of aplastic anemia following neoarsphenamine treatment. Whether or not this condition has any relation to an un-

usually long retention of arsenic by the bones (marrow) as observed by Bulmer (10), and resulting in an injury of the hematopoietic function, cannot be decided at present.

The relation of the chemical constitution of certain organic arsenicals to their action on the optic tract of rabbits was recently studied by Young and Loevenhart (87). These investigators found that trivalent and pentavalent arsenicals containing an amino group or substituted amino group in the para position to the arsenic produce optic lesions, whereas the corresponding ortho or meta isomers are devoid of this property. The reason for this difference in injurious action is not known.

A very striking feature of the pharmacological action of the arsphenamines is the great variation in *individual susceptibility* in experimental animals and patients. The fact that some individuals exhibit unusually severe reactions may have its reason in variations in technic in the preparation of the solution and administration of the drug. On the other hand, well controlled animal experiments in which these factors can be excluded, still reveal a great difference in individual susceptibility. The conclusion is therefore forced upon us that the individual susceptibility is probably also related to quantitative differences in the method of disposal of the drugs by the body of different individuals, such as differences in rate of oxidation of the arsenic, rate of excretion, etc. That the latter factor (rate of excretion) has a very pronounced effect on the toxicity of a great variety of different arsenicals was clearly demonstrated by Voegtlin, Dyer and Thompson (76). A very great increase in toxicity is noted in animals with ligated ureters in the case of those arsenicals which are excreted to a considerable extent by the kidney (pentavalent organic arsenicals, neoarsphenamine), whereas ligation of the common bile duct results in an increase of the toxicity of arsphenamine, the arsenic of which is eliminated very largely with the bile. Preëxisting pathological conditions of the excretory organs therefore may be partly responsible for the clinical occurrence of jaundice, diminution or suppression of urinary secretion, dermatitis and other untoward effects of the arsphenamines (46). Individual patients may also show an idiosyncrasy to one of the arsphenamines and not to others (Stokes and Behn (69) and others).

Recent reports indicate that arsphenamine dermatitis can be successfully treated by sodium thiosulphate (59), (47), and thiosinamine (27).

Before leaving the discussion of the pharmacological action in mammals reference should briefly be made to the *bio-assay* of the arsphenamines. Inasmuch as these drugs are not chemically pure compounds, it is necessary to determine their toxicity on animals before they are offered for clinical use. This was originally done in Ehrlich's Institute by establishing the maximum tolerated dose, injected subcutaneously in mice. Lots with a lower M. T. D. than the official requirement were refused release. The arsphenamines are now officially controlled in the United States by the U. S. Public Health Service through the Hygienic Laboratory, the toxicity test being carried out on standardized rats by means of intravenous injections under well controlled conditions. (See Roth (61), (62), and Lake (44).) In England and Germany mice are used for this purpose, the drugs also being injected intravenously. Experience has shown that this toxicity control is an efficient safeguard against the clinical use of abnormally toxic preparations. That the result of the toxicity test is very greatly influenced by all sorts of conditions, therefore requiring a perfectly uniform technic, will be obvious from what has been said concerning the pharmacological action of these drugs in the preceding pages.

The parasiticidal action. In his numerous papers Ehrlich (16), (17) always stated that all trivalent arsenicals, including the arsphenamines are directly toxic for trypanosomes and spirochaetes. This view certainly does not agree with Hata's observations (19), confirmed by Truffi and Sabbia (70), Bronfenbrenner and Noguchi (9) and others, that *Spirochaeta pallida* exposed *in vitro* to arsphenamine or neoarsphenamine in concentrations of 1:1000 does not show any loss of motility and that when such organisms are transferred to fresh culture media normal growth is obtained. Similar results can be secured with other spirochaetes and pathogenic trypanosomes. A simple calculation would prove that the drug concentration used in these test tube experiments far exceeds the concentration established in the body after the administration of a therapeutic dose. The question therefore arises: How does arsphenamine exert its powerful parasiticidal effect in the infected animal? Some workers have attempted to explain it by postulating a stimulating action of the drug on immune body formation. If this were true it would have to be assumed that the production of a sufficient amount of immune bodies would take place in the course of a few hours, as the parasites disappear with therapeutic doses in as short a time as 6 to 8 hours. A more reasonable explanation was advanced by Voegtlin and Smith (81), who were able to show by quantitative measurements

of the trypanocidal action in rats, that the action of all arsenobenzene derivatives is characterized by a fairly long latent period, during which there is no evidence of any parasiticidal action. This latent period is then followed by a gradual destruction of the parasites (fig. 2). The situation is totally different in the case of arsenoxide. There is no latent period, the injection of the drug being followed immediately by the rapid destruction of the parasites (fig. 2). As Neven (50) had previously shown that some arsenicals of the arsenoxide type (R·AsO) were trypanocidal *in vitro* in very high dilution (1:1,000,000) it was reasonable to explain the latent period observed with the arsenobenzene

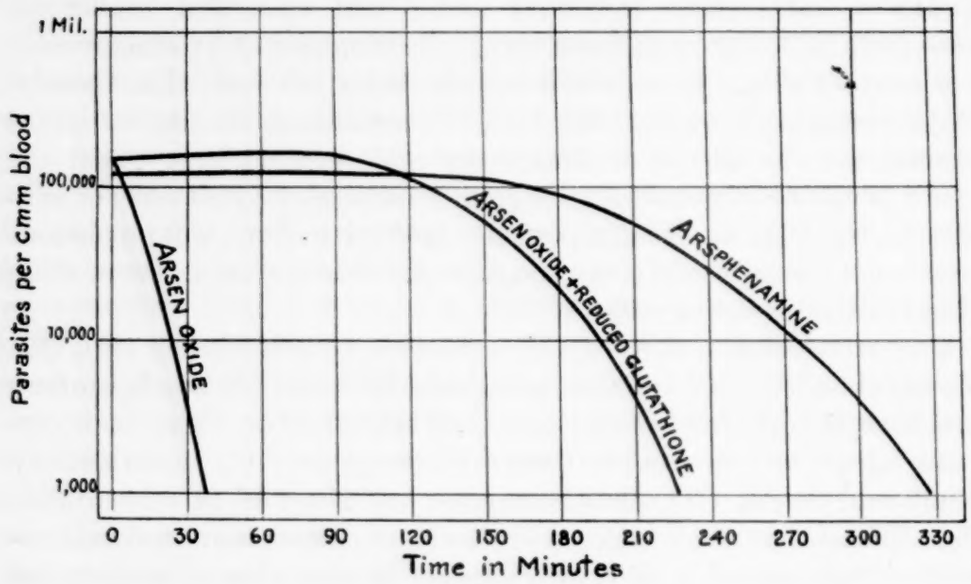


Fig. 2

derivatives as being due to the gradual partial oxidation by the host's organism of the As=As arsenic to the arsenoxide (R·AsO) form. Voegtlin, Dyer and Leonard (74) found that the trypanosome strain used in their work (*T. equiperdum*), is killed in a short time *in vitro* by a concentration of $\frac{M}{10,000}$ arsenoxide and a simple calculation shows that practically the same concentration of this drug is required in order to cause the disappearance of the parasites from the circulating blood of rats. Arsenoxide may, therefore, be regarded as the active parasiticidal modification of arsphenamine. Further evidence in favor of this view is furnished by the absence of a latent period after the injection of arsphenamine solutions which previous to injection have undergone partial oxidation *in vitro* by contact with air. These observations were recently confirmed by Simic (67) on trypanosome and *Spirochaeta*

recurrentis infection in mice treated with neoarsphenamine. In order to avoid any misleading results in the study of the action of neoarsphenamine on parasites *in vitro*, it is of course always essential to eliminate all possibility of oxidation of this drug before and during contact with the parasites.

Some unpublished work by the writer in collaboration with Doctor Johnson and Miss Dyer has shown that the trypanocidal action of the arsine ($R \cdot AsH_2$) derived from arsphenamine is also characterized by a latent period, a fact which is also in harmony with the above mentioned theory ($R \cdot AsH_2 + 2\ 0 = R \cdot AsO + H_2O$).

This theory is not in conflict with the following observations of Ehrlich (16), (17): If trypanosomes are exposed *in vitro* to arsphenamine and the parasites are then separated by centrifugalizing the suspension and further washing with serum, such parasites, as well known, retain their normal motility, but injected subcutaneously into mice have lost their pathogenicity. Ehrlich considers this fact as proof for the fixation of the drug by the parasites and the *direct action* of arsphenamine. We agree with Ehrlich that this experiment proves the fixation of the drug, which as previously pointed out, occurs also in the case of red blood cells and serum globulin. We believe, however, that the loss of pathogenicity is better explained by the oxidation of the fixed arsphenamine to arsenoxide by the tissues at the site of inoculation.

The crucial question now arises as to the mechanism whereby the parasites are killed by substances of the arsenoxide type. It was found (74) that this action can be explained in a similar manner as in the case of the toxic action of arsenoxide on mammalian tissues. Trypanosomes yield a nitroprusside test, indicating the presence of some sulphydril compound of unknown constitution. The addition of a certain minimum amount of various organic sulphydril compounds to a suspension of trypanosomes *in vitro* prevents the trypanocidal action of arsenoxide as shown by the survival of the parasites, indicated by the persistence of their normal motility. The following substances show this detoxicating effect: reduced glutathione, thioglycollic acid, thiolactic acid, thiosalicylic acid, cystein, glycyl-cystein, whereas various other amino acids and products of metabolism are totally inactive. A beautiful demonstration of the antagonistic action of the above mentioned sulphydril compounds on the trypanocidal action in the infected rat can be obtained by the intravenous injection of these sulphur compounds just before an injection of a minimum effective dose of arsenoxide. In this case the parasites do not seem to be affected for some hours (latent period in

fig. 2) and with a larger amount of sulphydril compound it is possible to prevent the parasiticidal action altogether. The mechanism whereby arsenoxide kills the parasites, therefore, appears to be essentially the same as the one responsible for the toxic effect of arsenoxide on mammalian tissue, i.e., an effect of the trivalent arsenoxide arsenic upon some sulphydril compound occurring in trypanosomes.

As will be recalled, the antagonistic function of sulphydril compounds on the arsenoxide toxicity in rats is at its optimum in the case of reduced glutathione and is less marked with cystein and thioglycollic acid. In other words it appears that only glutathione can completely protect mammalian tissues against arsenoxide injury and that cysteine and thioglycollic acid function less efficiently as oxidation-reduction catalysts in tissue metabolism. Trypanosomes, on the other hand, appear to be able to utilize a greater variety of organic SH compounds. Whether this different behavior of mammalian tissues and trypanosomes with regard to the specificity of SH compounds is due to the greater differentiation and the higher position in the evolutionary scale of the former cells can, of course, not be determined at present.

The parasiticidal effect of the arsphenamines is essentially related to the chemical toxicity (arsenoxide action) of these drugs, depending of course with different drugs to a great extent on the relative rate and amount of arsenoxide formation and the further oxidation of the latter to the pentavalent arsenicals. Compounds which are rapidly excreted (phenylarsonic acids) are generally less effective trypanocidal agents than the arsphenamines which are retained by the body of the host for a longer time (84). Arsenoxide shows the lowest rate of excretion of any of the organic arsenicals and has, therefore, the lowest minimum effective dose, the parasites being exposed to the action of the drug for a very long time. This view was confirmed by experiments (75) showing that blocking of the paths of excretion by ligation of the ureters or the common bile duct respectively, leads to a great increase of the parasiticidal action of the arsphenamines and related arsenicals.

Differences in the rate of *intra vitam* arsenoxide formation and its further oxidation to pentavalent arsenic and differences in the rate of excretion probably explain also the variations of the parasiticidal action observed in different animals of the same species and may also play a rôle in the differences of the parasiticidal potency of a given drug in different species of animals.

A further important factor concerned in the parasiticidal action, which has only in recent years been fully appreciated, is the *power of*

the drug to penetrate tissues. For instance, the absence of a pronounced therapeutic effect after intravenous injections of arsphenamine and neoarsphenamine in tabes and general paralysis is now attributed to a deficient penetration of the arsenicals into the central nervous tissues or to put it more correctly, the failure of establishing a sufficient concentration of arsenoxide within these tissues so as to cause the death of the parasites. Chemical determination of the arsenic content of cerebrospinal fluid and nervous tissue has shown that some of the arsenic reaches the central nervous system, but this method fails to give any information as to whether or not this arsenic is present in an active parasiticidal state (arsenoxide). A biological method, based on the destruction of trypanosomes inoculated into the cerebral subarachnoid space of rabbits following the intravenous injection of various arsenicals, was therefore applied to this problem by Voegtlin, Smith, Dyer and Thompson (83). They found that the parasiticidal effect was rather poor with even massive doses of arsphenamine, silver arsphenamine, neoarsphenamine and arsenoxide, and much better in the case of tryparsamide, atoxyl and sulpharsphenamine. The present clinical evidence appears to be in harmony with these experimental findings. The different rate of tissue penetration of the various arsenicals may perhaps be attributed to differences in rate of diffusion of the unaltered drugs into the tissues, for it happens, as mentioned in a preceding chapter, that atoxyl and related pentavalent arsenicals actually diffuse at a greater rate through semipermeable membranes than arsphenamine, neoarsphenamine and silver arsphenamine. Data on the rate of diffusion of sulpharsphenamine, arsenoxide, etc., are lacking.

A current misconception of the alleged superiority of the therapeutic effect obtained from intravenous injections of the arsphenamines, as compared with intramuscular and subcutaneous injections should receive passing attention. This view is contrary to all available experimental facts. Castelli (11) observed that neoarsphenamine injected subcutaneously is less toxic and more spirochaeticidal than when given intravenously. Voegtlin and Smith (82) obtained a similar difference in favor of the trypanocidal effect of the intramuscular over the intravenous injection of arsphenamine and neoarsphenamine. As previously pointed out the rate of excretion of the arsenic of these drugs after intravenous injection is greater than after intramuscular. With the latter mode of administration, the parasites are therefore kept for a longer time under the influence of arsenoxide and apparently it does not matter whether this active modification is partly produced at the place of the

subcutaneous and intramuscular injection respectively, the main point is that the parasites should be exposed to a sufficient concentration of arsenoxide for as long a time as possible.

If arsenoxide is really the parasiticidal substance formed from arsphenamine it might appear more desirable to use arsenoxide in therapeutics instead of arsphenamine. However, the margin of safety as established by the chemotherapeutic index $\left(\frac{\text{minimum lethal dose}}{\text{minimum effective dose}}\right)$ is somewhat lower in the case of arsenoxide than with arsphenamine and furthermore it has been shown by Hunt (30) that if even 10 per cent of the arsenic of arsphenamine is replaced by arsenoxide, typical arsenoxide symptoms are observed. Dale (12) explains the superiority of the arsphenamines over the arsenoxides as follows:

> If the ready-formed arsenoxide is injected, there will momentarily be present in the blood a concentration of the direct parasiticide higher than is ever produced by its slow formation from arsphenamine; but the arsenoxide is so toxic for the host that the concentration cannot in any case be raised to a high level with safety, and the dose given at one injection must be small. To obtain the optimum effect, by administration of the arsenoxide itself, it would probably be necessary to maintain for hours or days a continuous infusion of the substance in very high dilution. This slow, *persistent* supply of arsenoxide can be maintained for relatively long periods by injecting the arseno-derivative.

We agree with this explanation as far as it goes, but it has still to be explained why the host tissues can resist the toxic action of a *slow stream* of arsenoxide *better* than the parasites. The final explanation must be sought in a difference, probably quantitative, in the defense mechanism against arsenoxide of the mammalian tissues on the one hand and that of the parasites on the other. For instance, it is reasonable to conceive that the tissues might have a higher oxidation potential for arsenoxide than the parasites. If this were the case, the tissues would be able to convert arsenoxide into the nontoxic pentavalent phenylarsonic acid at a greater rate than the parasites. It is also possible that the permeability of the parasites for arsenoxide may be greater than that of tissue cells. Further work is needed in order to clear up this fascinating and fundamental problem.

Further work on the biochemistry of protozoa is also required in order to explain the interesting phenomena of *arsenic resistance* of trypanosomes discovered by Ehrlich and his collaborators (15). It was found that repeated treatment of infected animals with subcurative doses produced an increased resistance to the drug, which can become a

permanent property of the organisms. Voegtlin and Smith (81) produced an arsenic resistant strain of *T. equiperdum* by inoculation of the "normal" strain into rats which some days previously had received fairly large doses of arsphenamine or neoarsphanemine. A slight increase of arsenic resistance of *Spirochaeta pallida* was recently observed by Klauder (38) as a result of repeated treatment of syphilitic rabbits with subcurative doses of arsphenamine. Akatsu and Noguchi (1) had previously noted that *Spirochaeta pallida* acquires an increased resistance if the organisms are exposed to gradually increasing concentrations of arsphenamine in liquid media containing ascitic fluid and a piece of kidney. These results might be criticized on account of the well established fact that arsphenamine as such has no direct spirochaeticidal action *in vitro*. It must be remembered, however, that various workers (70), (73), (9) have observed that in the presence of tissues arsphenamine acquires spirochaeticidal properties, which are probably due to the formation of arsenoxide by the action of the tissues on arsphenamine. For the demonstration of an increased arsenic resistance *in vitro* it is best to work with compounds of the directly parasiticidal arsenoxide type, for it has been shown (75) that a trypanosome strain rendered resistant to arsphenamine in rats also shows a higher resistance to arsenoxide *in vitro* as compared with the normal strain from which the resistant strain is derived. These observations and similar ones by Neven (50) certainly indicate that the arsenic resistance noted in the injected animal is a characteristic of the parasite and is due at least to some extent to a greater effectiveness of the defense mechanism, whatever its nature, of the parasites against arsenoxide compounds. And this conclusion is in no way invalidated by the interesting observations of Mesnil and Brimont (48) and Breinl and Nierenstein (8) that the arsenic resistance of the parasites observed in one host species (mouse) may be much reduced or only slightly pronounced in another host (rat). Such differences may ultimately be explained on the basis of quantitative differences in the rate of excretion and in the changes which the drug undergoes (rate of arsenoxide formation) in the different hosts. Differences in the distribution of the drug among the tissues of different species may also play a part. Quantitative experiments of this nature and particularly experiments designed to establish the resistance of such strains obtained from different hosts (mouse, rat, dog) to arsenoxide compounds *in vitro* (always with proper controls of the normal strain) will probably assist in the solution of this problem of the rôle of the host species. It does not appear to me necessary to resort for this purpose

to a subsidiary hypothesis, such as the formation of Levaditi's (45) hypothetical trypanotoxyl. This author had found that atoxyl when incubated with an extract of liver is converted into a directly toxic modification, a fact which he interprets as showing that the drug is reduced to the trivalent arsenoxide, which, *only after combination with some tissue protein*, becomes directly toxic for trypanosomes. However, it is not at all necessary to postulate such a hypothetical trypanotoxyl, as arsenoxide itself is highly poisonous for trypanosomes and as Roehl (60) has shown that the trypanocidal power of the trivalent arsenoxide derived from atoxyl (p-amino-phenyl arsenious oxide) is reduced, instead of increased, in the presence of liver extract. This reduction of the trypanocidal potency of the arsenoxide by liver extract is, as we now know, very likely due to the presence of a certain amount of reduced glutathione in such an extract. Of course it can not be denied that p-aminophenylarsenious oxide—an amphoteric substance—may combine with the proteins of the tissues and of the parasites in a manner similar to arsphenamine, but the above observations indicate that such combinations are less active parasiticidal agents than the free arsenoxide compounds. With due consideration to these observations it was therefore suggested (75) that an increase in the arsenic resistance may perhaps be explained by an excess formation of sulphydril compounds by the parasites thus shifting the equilibrium $\frac{R\cdot SH \leftarrow S\cdot R}{R\cdot SH \rightarrow S\cdot R}$ toward the left.

A similar explanation may account for the reciprocal relation of resistance of trypanosomes to arsenic and quinoid dyes, discovered by Kudicke (1911) and Gonder (1912). In work which will shortly appear in the Journal of Pharmacology it was shown that the pharmacological action of at least one quinoid dye—methylene blue—is essentially due to an oxidation of the sulphydril group of the reduced glutathione of the rat tissues, the dye itself being simultaneously reduced to the leuco compound. Hence it is not improbable that treatment of trypanosomes with such dyes may affect the sulphydril system of the parasites in a similar manner as in the case of arsenic and this ultimately may explain this perplexing reciprocal relation between arsenic and dye resistance.

The arsphenamines are of practical value in the treatment of a great variety of protozoal and spirochaete infections, such as syphilis, yaws, relapsing fever, Vincent's angina, rat-bite fever, amebic dysentery, (ineffective in yellow fever). Suggestive results have also been obtained

in anthrax infection. The reason why arsphenamine treatment should produce a cure in one disease (relapsing fever) and not in another (yellow fever), though the causative organisms of both are closely related, is unknown. It is quite possible that differences in *1*, the chemistry of the parasites, *2*, the location of the parasites within the infected host, and other factors may play a rôle. It is also possible that in certain diseases, relapsing fever for instance, immunbody formation may assist the drug in combatting the infection.

From a practical standpoint the experimental *evaluation of the therapeutic potency* of remedies of this class is very important. Without going into any detail, a few remarks may assist the reader in forming an opinion of the present status of this subject. There is no question that the final, experimental test of the therapeutic potency of any arsenical must be made on animals infected with the organism against which the drug is to be used clinically. For instance, before a new arsenical is to be advocated in the treatment of syphilis it is only fair that the drug be previously tested on syphilitic rabbits. Recent observations however have shown that such experiments are extremely laborious. It is no longer right to consider, as Ehrlich and Hata have done, the disappearance of spirochaetes from the primary lesions, the healing of the latter and absence of clinical relapse for 2 or 3 months as proof of sterilization. Pearce and Brown (56) have shown that such animals may still carry a latent infection of the lymphatic system. Proof for the presence of organisms within lymph glands is furnished by inoculating such material into the testicles of normal rabbits in which primary lesions are thus produced with great regularity. Another method of testing the sterilizing effect was advocated by Kolle (40). This method is based on the fact that a syphilitic animal is resistant to reinfection with syphilitic virus, as shown by failure of the production of lesions at the site of secondary inoculation. Both methods have their value but in the writer's opinion the lymph gland transfer yields more convincing results. Kolle, using his method, has found that it is extremely difficult to sterilize syphilitic rabbits by even large doses of the arsphenamines, a positive result being only obtained at an early stage of the infection, i.e., if treatment is instituted not later than about 45 days after infection. These results were confirmed in some unpublished work in the writer's laboratory and harmonize with clinical experience. It has also been found that there is a great individual variation in the therapeutic effect of a given dose of a drug necessitating the use of a fairly large number of animals. The testing of drugs on syphilitic

rabbits therefore involves a very large amount of effort, and for this reason Schamburg, Kolmer and Raiziss (63) and Voegtlin and Smith (81) have advocated a preliminary test of the arsphenamines and related arsenicals on rats infected with *T. equiperdum*. The latter method especially permits to determine with considerable accuracy the parasiticidal potency on the basis of given numbers of parasites. As an example of the usefulness of such trypanocidal tests it may be stated that Voegtlin and Miller (80) have demonstrated the great difference in the trypanocidal value of neoarsphenamine of different manufacture. Dale and White (13) have confirmed these results and, what is very important, have shown that they agree with the spirocheticidal potency of the preparations in man.

Combination treatment. It was Ehrlich who first advocated the combination of several chemotherapeutic remedies in the treatment of disease. From the theoretical standpoint this method should possess certain advantages, inasmuch as it should permit to reduce the toxic action upon the host's organism (different distribution of the remedies in the body) without at the same time reducing the toxic action of each remedy on the parasites. The latter may succumb to the summation of the toxic effects of the several drugs, each given in smaller doses than those required to produce the same effect were each drug given alone. The combined treatment also may lower the chance of producing drug resistant strains. As is well known a combination of arsphenamine and mercury is the generally adopted method of the treatment of syphilis.

GENERAL CONSIDERATIONS OF THE CHEMOTHERAPEUTIC PROCESS AND THE CHEMORECEPTOR THEORY

The purpose of these concluding remarks is an attempt to bring some order into this great mass of somewhat disconnected facts which has been brought forth during the last fifteen years. It will be realized that this work has furnished a reasonable answer to some of the questions referred to in the introduction, such as the cause of the untoward reactions and the deficient sterilizing power of arsphenamine in certain diseases. It is also evident that the peculiar physical properties of arsphenamine, which appear to be the cause of some of the toxic reactions, are not at all essential for the therapeutic effect and future work should therefore concern itself to produce preparations devoid of these undesirable properties. Neoarsphenamine, sulpharsphenamine and tryparsamide appear to have certain obvious advantages over arsphen-

amine; though the latter has certain points of superiority, but the optimum drug for the treatment of syphilis, for instance, is still to be discovered.

The chemotherapeutic process is evidently of a very complex nature, involving to a great extent the participation of the host. Ehrlich has set a splendid example to others in not being satisfied with the mere discovery of valuable remedies. Instead, he has spared no effort in order to build up a theoretical foundation, which might serve the orderly progress in chemotherapeutic research. His chemoreceptor theory, largely based on his observations on drug resistance of trypanosomes, was supposed to give a rational basis for an understanding of the chemotherapeutic process. Briefly stated, this theory postulates the existence in both mammalian and parasitic protoplasm of chemical groups (side chains) attached to the so-called "Riesenmolekül or Biogen" of the cells. These chemical groups or receptors by chemical reaction are supposed to combine with food material as well as poisons. In the case of arsphenamine it is assumed that chemical fixation of the arsenic by the arseno receptor is essentially responsible for the toxic effect and the latter is enhanced moreover by the presence of the hydroxyl and amino group of the drug, which combine with some other hypothetical receptors (haptophore groups) of the cellular molecule, thus leading to a firmer fixation. The fact that arsphenamine in therapeutic doses kills only the parasites and not the host is explained by the assumption of a lower combining power (avidity) of the tissue receptors as compared with the receptors of the parasite.

In this form the theory is no longer tenable, for we now consider protoplasm not as a large single molecule but as a complex physicochemical system made up of a great variety of chemical substances, undergoing constant change but being adjusted to a certain (dynamic) equilibrium with regard to the various components. However, the evidence presented in this review clearly shows that there is a kernel of truth in Ehrlich's theory, for we may consider (with certain obvious reservations) the SH group as the arsenic receptor. There is considerable support for the idea that arsenicals can only exert their toxic action on protoplasm by being first converted by the host into the arsenoxide modification as follows:

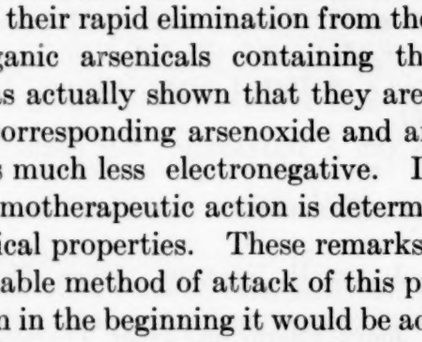
$$R\cdot AsO_3H_2 \longrightarrow \underset{\text{Arsenoxide}}{R\cdot AsO} \longleftarrow R\cdot As = As\cdot R \longleftarrow R\cdot AsH_2$$

The directly toxic action of the arsenoxides is essentially due to an

interference of cellular oxidations and reductions controlled by the system $\begin{matrix} R\cdot SH \rightharpoonup S\cdot R \\ | \quad . \\ R\cdot SH \leftharpoondown S\cdot R \end{matrix}$. Further experimental work is needed to explain the reason why arsenoxide is more toxic for the parasites than for the host. It is tentatively suggested that this difference may be due to a greater oxidation potential of the host's tissues for arsenoxide, permitting a conversion of arsenoxide, or its combination with sulphydril compounds, into nontoxic and easily excreted pentavalent organic arsenic, this resulting in a better protection of the host. A vast amount of work on the biochemistry of both host tissues and especially of the parasites is required to settle this point and new experimental methods must be devised for the solution of this fundamental problem. It is quite likely that the application of oxidation-reduction indicators will serve a useful purpose in this connection.

Another important problem presents itself when we attempt to explain the influence of the introduction of certain chemical radicles into the benzene ring on the biological behavior of aromatic arsenicals. Ehrlich's school has shown that the introduction of CH_3, halogen and SO_3H groups generally tends to lower the chemotherapeutic value of the resulting compounds, whereas the introduction of an amino group very often has a favorable effect. It was also found that arsphenamine has more favorable chemotherapeutic properties than its isomers, indicating that a mere shifting of the relative positions of the hydroxy and amino groups leads to a considerable change in pharmacological behavior. The mere designation of certain groups as dystherapeutic or eutherapeutic does not assist in the understanding of this problem. In the writer's opinion these problems may be attacked by a study of the physicochemical properties of a series of compounds with different substituting groups. It is well worthy of effort to determine for instance the changes in dissociation constants, degree of dispersion, solubility at various pH, resulting from certain substitutions in the benzene ring. Work in organic chemistry has brought out the significant fact that the introduction of CH_3, halogen and SO_3H groups generally tends to increase the acid dissociation constant of organic compounds, and conversely basic groups (such as NH_2) tend to lower it. Is it not probable that changes in dissociation determine to some extent differences in the distribution and rate of excretion of the compounds and consequently the chemotherapeutic behavior of different arsenicals?

Observations made in the writer's laboratory also indicate that sulphonated compounds



are so easily soluble in water at any pH that it is very difficult indeed to obtain the substances in pure form in the solid state, quite contrary to the behavior of the compounds containing an OH, NH_2 or substituted NH_2 group in place of the SO_3H group. Is the low toxicity of these sulphonated compounds perhaps due to their great water solubility which may result in their rapid elimination from the body? In the case of pentavalent organic arsenicals containing the strongly acidic—AsO_3H_2 group it was actually shown that they are much more rapidly excreted than the corresponding arsenoxide and arsenocompounds, in which the arsenic is much less electronegative. It is quite likely that the optimum of chemotherapeutic action is determined by an optimum of the physicochemical properties. These remarks are merely intended to indicate a reasonable method of attack of this problem. In order to simplify the problem in the beginning it would be advisable to determine the physicochemical properties of the directly active modifications, this being followed by a thorough study of the distribution and rate and mode of excretion and a proper correlation of the physical and biological data. In all events I believe that work of this nature with the arsenicals offers perhaps a better opportunity of furthering our knowledge concerning the relation of chemical constitution and pharmacological action, than is offered by most other drugs, and this for several reasons: 1, because the pharmacological action is certainly to a major extent an inherent property of the trivalent arsenic combined with oxygen (−As:O) and to a minor extent dependent on the chemical character of the organic radicles attached to the benzene ring; and 2, because we possess some evidence as to the chemical nature of the so-called receptor substance of living cells with which the − As:O reacts.

In conclusion I hope that this review will permit the reader to gather the essential experimental facts which have been discovered in this field during the last decade, and to recognize the obvious needs for further work on the nature of the chemotherapeutic process of the arsenicals. The speculative suggestions which were made of course can

only serve a useful purpose if they stimulate further experimentation and result in the discovery of new facts, which when properly correlated may perhaps again necessitate a modification or even reconstruction of the present altogether inadequate theoretical foundation of the chemotherapy of arsphenamine and related compounds. In all events, the extension of our theoretical knowledge is bound to lead, as it has led in the past, to important practical applications in the cure and prevention of certain diseases by these chemotherapeutic agents.

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BACTERIOPHAGE

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The following observation was reported by Twort (1) in 1915. In an attempt to cultivate ultramicroscopic organisms, agar plates were made from vaccine lymph from the calf. On certain of these plates, on which colonies of a micrococcus developed, "watery-looking areas" were observed from which subcultures could not be obtained. These areas later became glassy and transparent. Microscopic examination of films of material taken from the areas revealed only granules which stained reddish with Giemsa. When large quantities of the glassy material were spread on agar plates a few colonies of the micrococcus developed. These colonies soon became watery, glassy and transparent. When a bit of a watery colony was put on a young normal colony, the latter soon took the "disease." Old colonies were more resistant to the disease and dead colonies did not contract it at all. The disease-producing agent contained within the glassy material passed through Berkefeld filters and was still capable of producing the disease after being diluted a million-fold with water or saline. The agent was rendered inactive by heat at 60°C. *Staphylococcus aureus* and *albus* were slightly susceptible to the disease. Streptococci, tubercular bacilli and yeast were resistant. The disease could be passed from culture to culture ad infinitum, the etiologic agent continuously increasing in quantity, but it could not be propagated in the absence of living cultures. Similar observations were made with some intestinal bacteria.

Twort made several interesting suggestions concerning the nature of the etiology of this disease of bacteria: 1, that the active material might be produced by the micrococcus; 2, that the "virus" might be an ultramicroscopic organism, pathogenic for bacteria; 3, that it might be living protoplasm which formed no definite individuals; 4, or an enzyme with power of growth.

Twort's article attracted little attention at the time, possibly because it appeared during the World War or because of the title under which it was published—"An Investigation of the Nature of Ultramicroscopic Viruses."

In 1917 d'Hérelle (2) made a report under the title, "Sur un microbe invisible antagoniste des bacilles dysenteriques." The substance of this report was, that an ultramicroscopic organism which was actively antagonistic to the Shiga dysentery bacillus had been obtained from the stools of patients convalescing from dysentery. The ultra-microbe was given the name *Bacteriophagum intestinale* and was found to possess the following properties: 1, when put into young broth cultures of Shiga bacilli, growth of the culture was inhibited and the bacilli already present were dissolved; 2, the invisible microbe could be passed from culture to culture of the bacilli without suffering any loss of potency through dilution, i.e., it grew or multiplied; 3, it could not be propagated in the absence of living bacteria; 4, when it was mixed in proper proportion with a suspension of the Shiga bacilli and spread on agar plates, clear spots appeared in the film of bacterial growth; 5, when first isolated the ultra-microbe was virulent only for the Shiga organism but it acquired virulence in vitro for other bacteria; 6, it was harmless to animals but rendered them resistant to Shiga bacilli. From these and subsequent observations, d'Hérelle formed the conclusion that the ultra-microbe, *Bacteriophagum intestinale*, constitutes our chief defense against the ravages of pathogenic bacteria.

d'Hérelle's report aroused the interest of numerous investigators and hundreds of articles have since been published on the subject. That something can be obtained from the stools of patients convalescing from dysentery that will give rise to the various phenomena described by d'Hérelle has found universal confirmation but his conclusion as to the nature of this agent has not been generally accepted. In fact, the chief object of subsequent investigations has been to determine its nature.

The purpose of this paper will be to give briefly, and in a general way, the chief points of interest concerning the phenomenon now known, after its discoverers, as the "Twort-d'Hérelle phenomenon." Those interested in more detailed considerations of the subject are referred to a recent article by Otto and Munter (3).

It is quite evident that Twort and d'Hérelle were dealing with very similar or identical things. They had some substance or agent which was capable of bringing about the destruction of growing bacteria and at the same time of increasing or growing itself. Twort did not give a definite opinion as to the nature of this active substance but d'Hérelle, as stated above, believed that he was dealing with an ultramicroscopic organism that lived as a parasite on bacteria. This organism, accord-

ing to d'Hérelle's belief, secreted a ferment-like substance which was the immediate cause of the destruction of the bacteria, etc. This substance, regardless of its nature or source, is now generally known as bacteriophage or simply phage. These terms are used in this article in referring to the active agent concerned in the Twort-d'Hérelle phenomenon as if it were a definite entity or organism but without any commitment in regard to its nature or whether there is one or many varieties.

The source of phage. The agent phage has been found to be rather widely distributed in nature. Its chief source, however, seems to be the intestinal tract of man and the higher animals. It has also been obtained from pathological tissues, exudates, bacterial cultures, soil, water, etc. To determine whether a given material contains phage, or to obtain a specimen of phage for study, a small portion of the material is put into nutrient broth which is kept at 37°C. for one or more days. The contaminating bacteria usually form a luxuriant growth. The bacteria are removed by centrifugilization and filtration. The clear, sterile filtrate may or may not contain a phage active for one or more bacteria. This can be determined by adding some of it to young broth cultures of various bacteria and noting the effect upon these cultures; or by making agar cultures from them and noting the nature of the colonies that develop. If the broth cultures continue to grow normally and if the colonies on agar develop normally, it is indicated that the filtrate does not contain a phage active for the bacterium being used. The presence of a phage is indicated by inhibition of the growth and clearing of the broth cultures and the abnormal forms of the colonies on agar. The first cultures may not give any evidence of the presence of a phage but one may become evident if the sterile filtrate from the first broth culture is added to a second culture and from this to a third culture and so forth. Cultures on solid medium seem to be a more delicate test than fluid cultures.

Propagation of phage. When once a sample of phage is obtained, it can be propagated indefinitely by transferring a small portion of the culture on which it is acting to subcultures of the same or other susceptible bacteria. This is generally done by sterilizing the original culture by heat or filtration and adding some of the sterile medium or filtrate to young cultures of the bacterium being used. No one has been able to propagate phage in the absence of living, growing bacteria.

The growth of phage. The fact that phage can be passed indefinitely from culture to culture indicates that it grows or multiplies. Its power of growth can be definitely determined by experiment. For example, it may be found that 0.001 cc. is the smallest quantity of a given active filtrate that will affect the growth of a susceptible bacterium in 10 cc. of broth. The filtrate from this affected culture will be found to be just as potent, if not more potent, than the original filtrate, although the latter was diluted a thousand-fold in the second culture. Similar passages can be made indefinitely, usually with an increase rather than a reduction in the phagic power of the filtrates from the cultures.

There is a definite relationship between the multiplication of the bacteria and the increase in phage. When medium containing phage is inoculated with sensitive bacteria there is no increase in phage during the period of bacterial lag, but during the period of logarithmic growth of the bacteria, the phage rapidly increases and reaches a maximum concentration within from four to five hours. The bacteria in these cultures increase in number for from four to seven hours, depending upon the concentration of the phage in the culture, and are then rapidly lysed. There is no correlation between the destruction or disintegration of bacteria and the production of phage. It is evident, therefore, that phage is a product of bacterial growth, rather than of bacterial disintegration.

The action of phage upon bacteria. 1, Many bacteria become phage-producers or phagogenic when exposed to the action of a sample of phage; 2, bacteria grown in the presence of phage may become either more or less virulent for animals; 3, the cultural properties of bacteria may be altered; 4, the character of the colonies formed on solid medium may be affected; 5, the antigenic properties of bacteria are often modified by the action of phage; 6, bacteria are killed and dissolved

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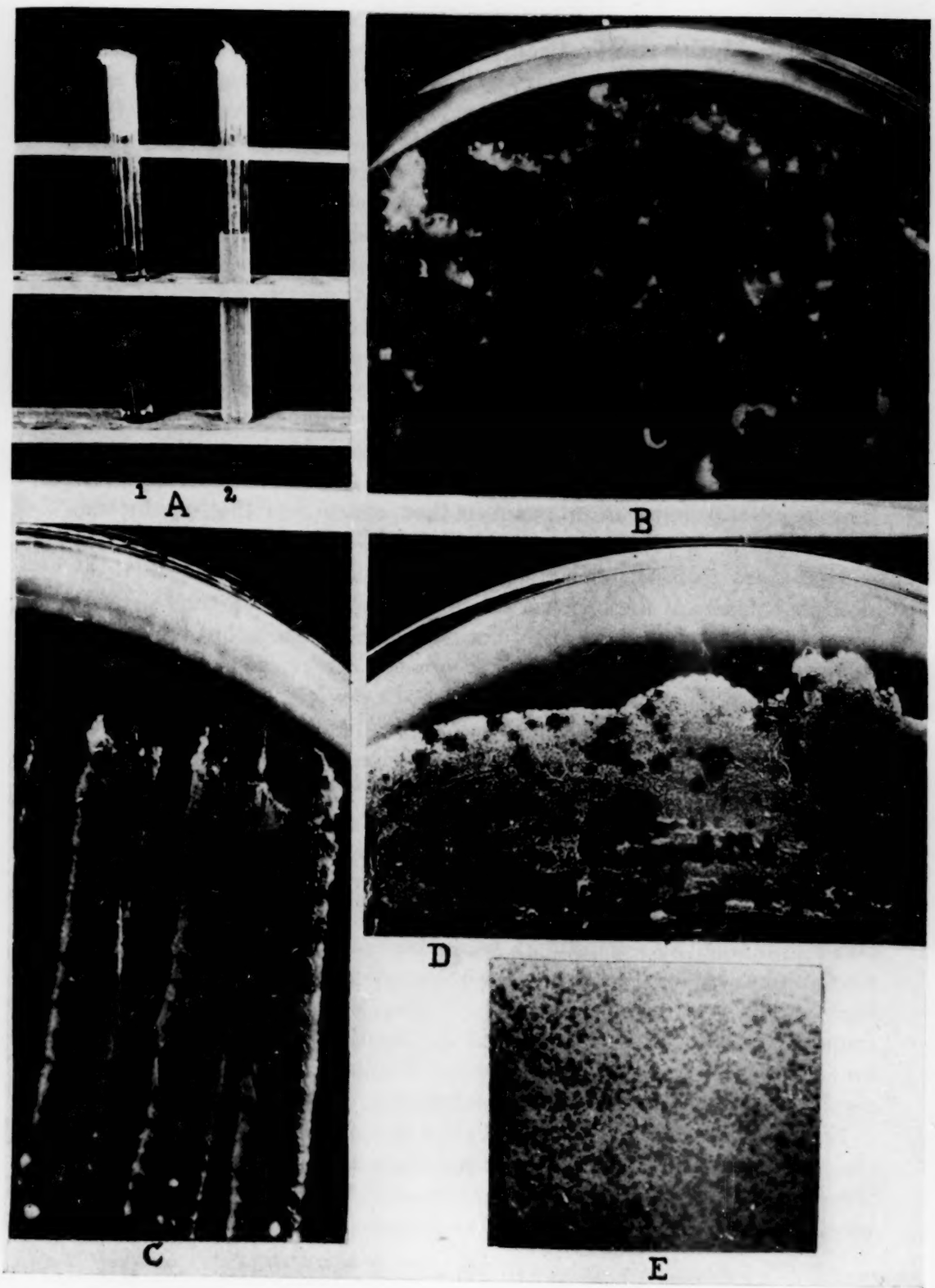
Fig. 1. A. Lysis of broth cultures. Two tubes of broth, *1* and *2*, were inoculated with Shiga bacilli and incubated until they had become turbid with bacterial growth. A drop of an active filtrate was added to tube *1*. Both tubes were returned to the incubator for 15 hours. Tube *1* had become clear, while tube *2* showed a heavy growth of bacteria.

B. Moth-eaten colonies. A solution of phage was added to a light suspension Shiga bacilli and a portion of the mixture was spread on the surface of an agar plate. The plate was photographed after 48 hours incubation.

C. Shows streak cultures of mixtures of phage and sensitive bacteria.

D. Large, irregular colonies of phage.

E. Small, circular colonies of phage.


1
A
2
B
C
D
E

by phage (see fig. A). Some of the characteristics acquired under the influence of phage are transmitted to subsequent generations.

Colonies of phage. If a suspension of bacteria and phage are mixed in the proper proportions and spread on agar, the film of bacteria growth developing on the agar will be "shot full of holes" (see figs. D and E). The holes result from the absence of bacterial growth in certain spots on the medium. The clear spots may vary in size and shape but they are usually relatively small and circular. These spots were designated by d'Hérelle as "colonies of bacteriophage," since, according to his interpretation, they result from the growth of the individual elements of the ultramicrobe, *Bacteriophagum intestinale.* For example, if a hundred clear spots develop on a given agar plate, the bacterial suspension from which the plate was made is supposed to have contained one hundred ultra-microbes which, being scattered over the surface of the agar, grew and dissolved the surrounding bacteria and, since the ultra-microbe is invisible, the clear spots appear. This point will be taken up again in the discussion of the hypotheses on the nature of phage.

Moth-eaten colonies. When bacteria and phage are mixed so that discrete colonies of the bacteria develop on solid medium, the colonies often exhibit various bizarre formations, such as halfmoons, cresents, etc., with very irregular edges. It appears as if the colonies were being "nibbled away" by an invisible foe. Such colonies have been designated as "nibbled" or "moth-eaten" (see figs. B and C). Samples of phage that are too weak to influence the growth of bacteria in fluid medium will often give rise to moth-eaten colonies.

Bacteria sensitive to phage. The different bacterial species manifest varying degrees of susceptibility to the action of phage; some species being extremely susceptible, while others are apparently absolutely resistant. Even pure cultures of bacteria have been found to be composed of individuals exhibiting a wide range of resistance or sensitiveness to the action of phage. The most sensitive bacteria belong to the typhoid-dysentery-colon group. Other bacteria that have been found to be more or less subject to the bacteriophagous phenomenon are: the diphtheria bacillus, the plague bacillus, the bacillus of hemorrhagic septicemia, staphylococci, streptococci, the cholera vibrio, etc.

Virulence of phage. Notwithstanding the fact that the nature of phage has not been definitely determined, it is convenient to use the term "virulence" to express its power to attack and destroy, or otherwise affect, bacteria. The virulence of a sample of phage for a given

strain of bacteria is measured by determining the smallest amount that will inhibit or otherwise affect the growth of the culture. We have had strains of phage that were so virulent for a certain strain of B. dysenteriae (Shiga) that cultures would not develop in broth containing one-trillionth part of the filtrate from a culture on which the phage had been allowed to act.

Variation in the degree and range of virulence constitutes one of the most interesting characteristics of phage. As a rule, samples of phage obtained from natural sources are virulent for more than one kind of bacteria but exceptionally they may affect only a single bacterial species. Both the range and degree of virulence may be modified by artificial means, such as "passage" in vitro with different kinds of bacteria. A specific case will serve to illustrate this point. A strain of phage was obtained from the excrement of chickens which was, at the time of isolation, extremely virulent for a certain strain of B. coli. It also manifested a slight degree of action on strains of B. dysenteriae (Shiga and Flexner), B. typhosus and B. paratyphosus, A and B. It was propagated in cultures of the Shiga bacillus for eighteen months. It no longer affected cultures of the colon bacillus but was exceedingly virulent for the Shiga organism. Its power to affect the other bacteria had not been appreciably altered.

Resistance to heat, chemical reagents, etc. The agent phage has been found to be extraordinarily stable. It remains potent for a number of years when kept in sealed tubes at room temperature. Most specimens of phage are not rendered inactive by heat below 75°C. Twort found that 60°C. for thirty minutes destroyed his transmissible lytic agent. Others claim to have had specimens of phage that were not destroyed by temperatures under 100°C. A polyvalent phage (one which affects more than one species of bacteria) may be rendered inactive for one species of bacteria and retain its virulence for other species. There seems to be no doubt that there is a wide variation in the heat resistance of different specimens of phage, the range being from 60°C. to 75°C. or higher. Even the same strain of phage seems to vary from time to time so that it is difficult to establish a definite "thermal death-point" for a particular strain. As a rule phage is more resistant to heat than are the bacteria on which it grows, thus making it possible to separate them by means of heat.

Phage is much more resistant than bacteria to a number of chemical antiseptics. For example, it is not killed by an excess of chloroform, acetone or alcohol. It has been found to withstand 2.5 per cent phenol,

1 per cent copper sulphate, 2 per cent lysol, 2.5 per cent sodium floride, and high percentages of other antiseptic reagents. It is, however, rather susceptible to acids, alkalies and quinine hydrochloride. Proteolytic ferments do not destroy it.

BACTERIOPHAGE AND IMMUNITY. *Anti-phage.* Bordet and Ciuca (4) were the first to show that an antibody can be obtained for bacteriophage. If rabbits are given a series of injections of fluids containing phage or of phage-producing bacteria, their sera acquire the power of neutralizing the action of phage. The immune serum is tested by mixing it with an active phage, allowing the mixture to stand at 37°C. for thirty minutes and letting a drop or two of it flow across the surface of agar which has been inoculated with sensitive bacteria. If the phage has been neutralized the entire surface of the agar will be covered with bacterial growth, while if the phage is still active, the path of the drop will remain free of bacterial growth. As a rule, the anti-serum is not very potent, only low dilutions show activity and it may be necessary to dilute the solution of phage in order that the effects of the serum may be manifested. The anti-serum is effective only against a phage that has a range of action corresponding to the one used to immunize the rabbits. For example, a serum produced with an anti-Shiga phage does not neutralize a phage active for staphylococci but does neutralize an anti-colon phage, provided that the phage used as antigen was active for the colon bacillus. The anti-phage is of the nature of an antitoxin.

The immune serum obtained by treating animals with preparations of phage contains various anti-bacterial antibodies (precipitins, agglutinins, opsonins, etc.), since every preparation of phage necessarily contains bacterial products. These antibodies can be removed from the serum by absorption with bacteria without removing the anti-phage. The absorbed sera still neutralize the action of phage and fix complement in combination with preparations containing phage but not with bacterial preparations which do not contain it. d'Hérelle claims that the immune sera contain an antibody demonstrable by complement-fixation, which is specific for the ultra-microbe, *Bacteriophagum intestinale*, but other investigators have been unable to demonstrate the presence of such an antibody.

d'Herelle's theory of immunity (*5*). The discovery of phage has led d'Hérelle to formulate a new theory of immunity, the most important factor of which is the ultra-microbe—*Bacteriophagum intestinale.* According to this theory the mechanism of immunity consists of three

factors: 1, *Bacteriophagum intestinale;* 2, cellular immunity; and 3, antitoxin immunity. The ultra-microbe acts in three ways: *a*, through direct antagonism for the disease-producing agent; *b*, the preparation of bacteria for phagocytosis; and *c*, rendering the bacteria particularly potent as antigens. Thus phage plays a particularly important rôle, for, besides acting directly and independently against the pathogenic agent, it is indirectly concerned in the other two factors. A concrete case will help to gain a clear conception of the theory. When typhoid bacilli succeed in entering our tissues, one of four things follows: 1, If we are fortunate enough to be carrying a phage virulent for the typhoid bacilli, the invading organisms will be attacked and destroyed by the phage before they have had time to multiply and cause an infection. In this case no disturbance arises and we remain unaware of the coming and going of the bacilli. Theoretically, a degree of immunity should arise as a result of the action of antigenic substances derived from the destroyed bacilli. 2, We may be carrying a phage which is not actively virulent for the invading bacteria which, unopposed, give rise to infection and disease. In the meantime, the phage acquires virulence for the infecting bacteria and destroys them before the host is overwhelmed. The disease is thus checked and the patient recovers. 3, The phage may be able to oppose the bacilli sufficiently to prevent disease but not be able to destroy them. It is thus that typhoid carriers arise. 4, If the phage is absent, or present and unable to interfere with the growth of the bacilli, a fatal infection develops.

It will not be necessary to present the detailed observations upon which d'Hérelle has founded his theory of immunity but he maintains, in general, that in all of the infectious diseases thus far studied the severity of the infection has been inversely proportional to the virulence of the accompanying phage for the bacteria causing the infection. A similar statement could be made concerning susceptibility and resistance to infection. d'Hérelle's observations were made chiefly on dysentery and typhoid in man, fowl typhoid, and hemorrhagic septicemia in cattle and buffalo.

Phage as a preventive and curative agent. Having arrived at the conclusion that resistance to infection and recovery from infectious diseases are dependent upon the presence of a strain of phage virulent for the bacteria concerned, the next step was to supply the proper phage wherever it was lacking. This d'Hérelle did with universal success.

The following observations illustrate the results obtained with fowl typhoid: A, "The epizootic began on about August 20. By October 6, 120 of 200 fowls had died. The 80 survivors, of which seven were sick, received an injection of anti-gallinarium bacteriophage. The seven sick fowls recovered and the epizootic immediately and permanently disappeared." B, "On February 23 the surviving chickens of a poultry-yard containing at that time 102 animals were immunized. The epizootic which began about ten days previously, and which had resulted in a daily mortality of four or five chickens, stopped quickly and permanently from the time of the immunization. The epizootic continued, on the contrary, to ravage with the same intensity as formerly in all the neighboring poultry-yards which served as controls" (0.5 cc. of a potent phage was injected subcutaneously).

Even more striking results were obtained in the immunization of cattle and buffalo against hemorrhagic septicemia. This disease is very acute and fatal, death occurring as a rule within 48 hours after infection takes place. A phage virulent for the organism causing this disease was obtained from an animal that had passed through an epizootic unaffected. Preparations of this phage were used in a series of artificial immunization experiments. The results were briefly as follows: A subcutaneous injection of 0.04 cc. of phage rendered the animals resistant to infection after one to five days; 0.25 cc. after 20 days; 20 cc. after 40 to 60 days. From 5 to 2000 lethal doses of the virus were used to test the resistance of the treated animals.

It is seen that the smaller the dose of phage, the sooner the development of immunity. For example, a dose of 0.04 cc. gave almost immediate immunity, while a dose of 20 cc. gave immunity only after from 40 to 60 days. d'Hérelle explains this peculiar result by assuming that injections of phage both sensitize and immunize the animals. When large doses are given, sensitization runs ahead of immunization for the first 40 to 60 days, while the sensitization following small doses soon disappears and the process of immunization is given full play. It is of interest to note also that the appearance of the immune state is very sudden, an animal which is highly susceptible one day may be able to resist a thousand lethal doses of the virus on the following day. The refractory state may continue for at least 14 months.

d'Hérelle also reports very favorable results in the treatment of bacillary dysentery. A number of cases are reported which practically recovered within 48 hours after the administration of 2 cc. of an appropriate preparation of phage, i.e., there was a cessation of bloody

stools, disappearance of the bacilli from the stools, prompt improvement in the general condition of the patient and uninterrupted recovery.

According to d'Hérelle's reports, bacteriophage leaves nothing to be desired in the prevention and cure of infectious diseases but one gets the feeling that "it is too good to be true." In all probability this will prove to be the case, for, with the exception of reports by Beckerich and Hauduroy (6) on the treatment of typhoid; and Gratia, and Bruynoghe and Maisin (8) on staphylococcus furunculosis, no one has been able to obtain results which in any way confirm d'Hérelle's conclusions. Davison (9) had absolutely negative results in the treatment of dysentery in children and Otto and Munter (3) report negative results in the treatment of both typhoid and dysentery.

Final judgement on phage as a preventive and therapeutic agent must await further observations.

THE NATURE OF PHAGE. Twort did not come to any definite conclusion as to the nature of the active principle concerned in the phenomenon which he described but it is of interest to note that the leading suggestions made by him have been embodied in the hypotheses formulated by later investigators: 1, That the causative agent is a part of, or is secreted by, the bacterium affected. He says, "If it is a part of the micrococcus it must be either a stage in its life-history which will not grow on ordinary media but stimulates fresh cultures of the micrococcus to pass into the same stage, or an enzyme secreted by the micrococcus which leads to its own destruction and the production of more enzyme." 2, That the causative agent is a separate form of life—an ultramicroscopic virus. Concerning this possibility he says, "In any case, whatever explanation is accepted, the possibility of its being an ultra-microscopic virus has not been disproved—."

d'Hérelle early came to a definite conclusion concerning the nature of phage and has since found no reason for altering his original hypothesis. According to this hypothesis, the agent to which the bacteriophagous phenomenon is directly attributable is an enzyme secreted by an ultramicroscopic organism—*Bacteriophagum intestinale*. This ultramicrobe is believed to be an obligate parasite of living bacteria and cannot multiply except in the presence of growing bacterial cultures. It penetrates the bacteria and grows at their expense. As a result, the bacterial bodies are broken up and dissolved.

Further observations led d'Hérelle to conclude that the ultramicrobe is a normal inhabitant of the intestinal tract of many, if not all, animal

species. Ordinarily it lives at the expense of saprophytic bacteria, but possesses a potential virulence for many pathogenic bacteria. When the latter enter the intestinal tract or invade the tissue of the animal, whether or not infection and disease ensue depends upon the readiness with which the ultra-microbe acquires the ability to attack and destroy the invading bacteria. In case *Bacteriophagum intestinale* is not present, infection, disease and death are almost sure to follow, for the infected individual has little other means of defense. If the ultra-microbe is slow in acquiring virulence for the invading bacteria, infection and disease may arise but finally the ultra-microbe successfully meets the emergency and the afflicted individual recovers. It often happens, especially when a particular disease is prevalent, that many individuals acquire a strain of phage already virulent for the bacterium causing the disease. These individuals do not contract the disease because the virus is destroyed before infection can occur. When a virulent phage becomes disseminated throughout an infected community, an epidemic necessarily ceases. The final step in the hypothesis is that infectious diseases can be prevented and cured by the artificial administration of the proper strain of *Bacteriophagum intestinale*.

A detailed description of the evidence offerred by d'Hérelle in support of the living virus hypothesis will not be undertaken here but the chief points in his argument may be briefly stated. 1, The bacteriophagous agent grows, because it can be passed indefinitely in serial cultures (of bacteria) without suffering a diminution in potency, notwithstanding the fact that the original cultures undergo unlimited dilution. 2, The virus exists in particulate form, because it is possible to determine the number of bacteriophagous elements in any given culture by counting the "colonies of phage" that develop on plates made from mixtures of phage and bacteria. 3, The active agent manifests the property of adaptation (variation) as shown in the acquisition of virulence for bacteria and increased resistance to chemical agents. 4, That it has been established by direct observation that the ultra-microbe penetrates bacteria, multiplies within them and thereby effects fragmentation and dissolution of the bacterial bodies. 5, That the ferment secreted by this organism can be separated from the ultra-microbe itself. 6, When a strain of bacteriophage is injected into appropriate animals an antibody is produced which fixes complement in the presence of any and every strain of phage but never in its absence. In other words, an antibody specific for the ultra-microbe

has been discovered, hence the microbe must exist. 7, That the decisive rôle which the ultra-microbe plays in resistance to infection and recovery from disease has been firmly established by clinical and experimental observations.

d'Hérelle maintains that the above observations admit of but one interpretation, namely: that the bacteriophagous phenomenon is the manifestation of the activities of an invisible organism, a parasite of living bacteria. He also states that the authors who have not accepted his interpretation have failed to consider all of the established facts.

As a matter of fact, the ultra-microbe hypothesis has been accepted by but few, while others have vigorously opposed it. It is maintained on the one hand that the various phases of the phenomenon can be satisfactorily explained without assuming the presence of an invisible organism and on the other that the agent concerned here exhibits properties which are incompatible with life. It must be admitted, however, that few, if any, of the counter hypotheses are more plausible or less fanciful than the ultra-microbe hypothesis.

Kabéshima (10) was one of the first to question the living nature of the bacteriophagous agent. This author cannot reconcile the heat-resistance and insusceptibility to poisons manifested by phage with living matter. It will be recalled that a definite "thermal-death-point" has not been established and that one specimen of phage may not be "killed" at 75°C. for 30 minutes, while another is rendered inactive at 60°C. Strains resisting 90° and 100°C. have been reported. A majority, however, become inactive when heated at 75° for 30 minutes. Such resistance to heat may be unusual but is it incompatible with life? Bacterial spores are more resistant, while non-sporing forms are as a rule less resistant. The insusceptibility of phage to protoplasmic poisons seems to be much more significant. As already mentioned, phage is not killed by an excess of chloroform, acetone, alcohol and is unusually resistant to sodium fluoride, phenol, copper sulphate, etc. Having, for these reasons, rejected the ultra-microbe hypothesis, Kabéshima advances his own hypothesis which may be stated briefly as follows: A catalytic agent, arising in the tissues of an infected animal, activates a proferment contained within the bacterial bodies; the activated ferment lyses the bacterium from which it arises, is then liberated and passes on to other bacteria to activate the proferments which they contain. Since this hypothetical catalytic agent acts as a defense against infection, Kabéshima suggests that it be named, "Ferment d'immunité bacteriolysant." It is main-

tained that this hypothesis is in accord with all observed facts and affords a plausible explanation of the apparent multiplication and infective power of the bacteriophagous agent. It is evident, however, that it is founded on pure assumption.

Bordet and Ciuca (1) came to the conclusion that the bacteriophagous phenomenon is to be ascribed to an hereditary nutritive vitiation of bacteria. The vitiation, it seems, consists of an exaggerated propensity toward autolysis. This hypothesis implies that the whole phenomenon may be looked upon as the manifestations of a nutritional disease of bacteria, which is not only inheritable but contagious as well. The disease, it is claimed, first arises as the result of the action of defensive agents (particularly leucocytic exudates) of an infected host upon the invading bacteria. This nutritional disorder brings about an autolysis of the affected bacteria and the liberation of an agent capable of transmitting the affection to other bacteria, while the affected bacteria multiply and transmit the disease to their offspring.

The Bordet-Ciuca hypothesis is based upon the following observation: A guinea pig was given three intraperitoneal injections of colon bacilli at intervals of five or six days. Two days after the last injection a few drops of the peritoneal fluid were removed and added to a broth culture of the same organisms. The culture medium soon cleared up. Subcultures from this tube produced a lytic agent which dissolved other cultures and the bacilli themselves had acquired a nutritional disorder (exaggerated autolysis) which was transmitted to subsequent generations.

It has not been possible to repeat the Bordet-Ciuca experiment with regularity and bacteria have been known to acquire the bacteriophagous disease without coming in contact with animal tissues or fluids. It appears, therefore, that their observations have no decisive significance and should not be taken as the basis of an hypothesis concerning the nature of the phenomenon.

Bail (12) published the following hypothesis: Under the influence of various agencies, especially the tissues and fluids of the animal body, bacteria undergo a peculiar kind of fragmentation which results in the liberation of small particles of the regenerative substance (chromosomes?) of the cell. These particles are called "Splitter." Of the two functions of the normal generative substance, building-up and tearing-down, the "Splitter" are capable of doing the latter only. When the "Splitter" come in contact with multiplying bacteria, they react with the generative substance of the growing cell and more

"Splitter" are formed. The bacteria from which "Splitter" are given off do not necessarily die but may live and reproduce as mutants, the power to react with this particular kind of "Splitter" having been lost. The "Splitter" are capable of living and reproducing at the expense of normal bacteria. They may also be pathogenic, constituting a filterable virus. For example, the etiological agent of typhus fever is probably the "Splitter" from the Weil-Felix proteus bacillus. Whatever the nature of the bacteriophagous agent may be, Bail maintains that it arises from the bacteria themselves.

Otto, Munter and Winlker (13) have a slightly different notion. They believe that the bacteriophagous agent consists of minute particles derived from the bacterial bodies, which are of themselves capable of bringing about fermentative effects or carry ferments with them.

A very interesting explanation of the bacteriophagous phenomenon has been made by bringing this phenomenon in alignment with the occurrence of bacterial variants—Eastwood (14), Arkwright (15). It is a well-established fact that variants or mutants frequently arise in bacterial cultures and also under natural conditions. These variations may be in many different directions, such as 1, morphology of colonies; 2, stability of suspensions in salt solutions; 3, serological properties; 4, fermentation of carbohydrates; 5, virulence; 6, toxin production, etc. It has also been possible to induce bacteria to form variants of a particular kind by subjecting them to the influence of particular agents, such as immune serum, temperature, reaction of medium, etc. The occurrence of variants among bacteria is, in all probability, the result of definite influences or stimuli. This statement is justified by the fact that particular types of variants have been observed to arise regularly when cultures are subjected to a fixed set of conditions.

Starting from this point it is not a long step to the bacteriophagous phenomenon, since one of the most characteristic properties of phage is the power of causing variants in the bacterial culture on which it is allowed to act. For example, cultures acted on by phage almost always give rise to sensitive and resistant strains. Each of these strains may or may not be phage-producers and they generally show variation in colony formation, virulence, etc. Phage-producing strains have also been obtained from cultures without the intervention of previously formed phage. Hence it is possible that as the result of some particular stimulus—animal tissues or fluids, or bacterial products—bacterial variants having exaggerated autolytic properties

arise. As a result of the autolysis, a substance is formed having the power to stimulate the formation of other variants in the same direction. In this way the stimulating substance becomes concentrated and the variation becomes more and more pronounced, finally reaching the advanced stage seen in the Twort-d'Hérelle phenomenon. This interpretation of the bacteriophagous phenomenon is based upon anology with observed phenomena and is to that extent satisfactory.

It must be confessed, however, that any of the various interpretations of the Twort-d'Hérelle phenomenon requires the assumption of phenomena which are as yet unproved and for which we have no exact anology. Final decision as to the nature of bacteriophage must, therefore, await the production of further evidence.

SUMMARY

The active agent, bacteriophage, occurs in the excreta of man and the higher animals, in water, soil, bacterial cultures, etc. It is characterized by 1, unusual stability and resistance to heat and antiseptics; 2, the power of killing and dissolving and otherwise affecting living bacteria; and 3, the power of reproducing itself in the presence of growing bacterial cultures.

Notwithstanding the very decisive results that d'Hérelle obtained with the preventive and therapeutic application of bacteriophage, the failure of others to obtain confirmatory results makes an open question of the relation of the bacteriophagous phenomenon to infection and resistance.

It is not possible at the present time to make a final decision concerning the source and nature of the bacteriophagous agent. The ultra-microbe hypothesis has not been satisfactorily established and none of the various other hypotheses has escaped the necessity of assuming unusual phenomena in order to explain the behavior of this agent.

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MORPHOGENETIC FACTORS IN THE DIFFERENTIATION OF THE NERVOUS SYSTEM

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The agencies which operate in the differentiation of the nervous system of the individual animal body are open to direct study by observation and experiment. Attempts have also repeatedly been made to reconstruct the phylogenetic history of this differentiation and its mechanism, such as those of Hertwig (30), Child (12, pp. 193, 464; 14, pp. 133, 210), Parker (44) and the present writer (29).

The indications are that well-defined nervous tissue early in phylogeny took the form of a non-synaptic nerve net (as in coelenterates) and that within this syncytial net the neurons were later set apart by the differentiation of synaptic junctions between the cellular elements of which the network is composed. In fact, we find in certain lowly organized mollusks and worms a nervous system which appears to be in part a nerve net and in part of synaptic type, a condition which seems also to persist in some sympathetic ganglionated plexuses of the human body (1).

In the central nervous system of the most primitive vertebrates, as in cyclostomes (46), and in larval Amphibia (personal observations) much of the tissue is composed of neurons of very generalized form without clear distinction of axonic and dendritic processes. We may picture such tissue as a collection of incompletely polarized neurons with relatively short equipotential processes which are much branched, so organized that excitations may be widely diffused through the nerve centers with incomplete isolation or insulation of particular functionally specific nervous pathways.

In the nervous systems of urodele Amphibia, both larval and adult, I have found many such generalized forms of neurons. In some cases there are numerous short processes arising from the cell body without morphological difference between dendrites and axons. The anatomical evidence suggests that such elements may conduct indifferently in any direction. In the olfactory bulb I have described (28) granules which seem to be unpolarized and also various forms of intermediate cells

between these granules and neurons of more conventional types with clear differentiation between dendrites and axons. Other intermediate forms of neurons are found.

The main lines of central conduction—the chief tracts of through traffic—appear to have been differentiated within such a relatively equipotential system, as Sherrington expresses it, by the sheer process of heavy traffic, as a tangle of country lanes or crisscross tracks on an open plane may be changed into a trunk highway by the growth of two cities out of cross-roads settlements and the wearing out of a road between the cities.

This appears to be the history of the evolution of the higher brains, with their more or less precisely localized centers, each concerned with some specific function and all connected by well-defined internuncial pathways which may be activated, now in one pattern, now in another, as evoked by fluctuating physiological processes. But how these obviously adaptive arrangements have come into being is not revealed by such observations.

In individual development the most frequently used fibers grow by use, the axons are elongated in the direction of most frequent transmission, the appropriate synaptic junctions assume the characteristics of lowered resistance, attunement, or whatever it is that facilitates transmission, while the collateral pathways remain relatively obstructed. The nerve centers take shape and their neurons assume their definitive structure under the influence of the traffic, much as a big switch yard of a growing railroad system gradually builds up, with its complement of tracks, freight houses, etc.

This is the prevalent idea of the way in which the nervous system grows during function, but we have few unambiguous observations which clarify the details of the process or explain its mechanism. Those that we have suggest that all learning has an anatomical phase which, if our technique were more refined, we could see in the form of outgrowth of new fibers for associational connections, of refinement of synaptic junctions at strategic points in the meshwork of cells and fibers, of change in the internal organization of the cell bodies as their specific functions are perfected (11, p. 887).

Embryologically the nervous system is not elaborated from a strictly equipotential nerve net or nervous feltwork, nor do we find such a condition in even the most primitive known types of adult nervous system. In all of these cases the nervous system is laid down within the preëxisting body gradients (14) and it has from the start some measure of

functional polarization. In the less highly specialized parts of the nervous system, even in mammals, there are primary physiological gradients of the same type as those investigated by Child in polyps and flatworms that still play an important rôle in the normal course of functional activity. This is brought out most clearly in Alvarez's studies (1), (2) of the physiological gradients of the intestine.

Though the primitive nerve net appears to persist in some peripheral ganglionated plexuses of mammals, it is not probable that this grade of organization is present at any stage of development of the central nervous system, the credentials of the plasmodesma of Held and others having doubtful validity. Certainly the nervous elements as soon as they are capable of functional activity are connected in synaptic chains whose arrangement is obviously from the first shaped with reference to the performance of adaptive movements, as has been shown most clearly by the researches of Coghill (15), (16). Yet in the earlier stages of all species, as in the adults of the more primitive vertebrates, the links of these neuronic chains are short and the long tracts which carry the through traffic of more highly specialized forms appear rather late in ontogeny. There are other similarities between phylogenetic and ontogenetic differentiation to which reference will be made at the close of this review.

In the embryological development of the nervous system the following factors are believed to be operative, and some or all of them may have influenced the course of phylogenetic development: 1. The hereditary organization of the body protoplasm. 2. The primary physiological gradients (Child) which can be clearly demonstrated in young vertebrate embryos, the regions of most rapid growth being the high points of the gradients. 3. Bioelectric factors arising from ordinary growth and differentiation and in other ways. 4. The development of blood vessels and other transportative mechanisms of nutrition of tissue. 5. Hormone action, including chemotaxis of one sort or another, expressing itself as an attraction to certain regions of the growing tips of the nerve fibers in a selective way. For such differential susceptibility there is some experimental evidence. 6. The activating effect of ingrowing nerve fibers upon a field which is in process of differentiation. 7. The effect upon surrounding parts of the functional activity of such nerve fibers as have already attained sufficient maturity to form parts of closed reflex circuits.

The first of these factors will not be considered here. The physiological gradients have been fully discussed in the works of Child (13),

(14). The action of the gradients in the process of embryologic differentiation of the spinal cord of Amblystoma is strongly suggested by some observations of Detwiler (26), where the interpolation of a piece of medulla oblongata in the cervical cord results in hyperplasias which apparently are direct results of the introduction of new centers of dominance in atypical places. Some other transplantations of spinal cord segments which are as yet unpublished (personal communication from Doctor Detwiler) give further support to the supposition that intrinsic gradients affect the rate of growth of the spinal cord.

Regarding the action of the third, or bioelectric, factor during the growth and differentiation of the vertebrate nervous system our actual knowledge is very slight, though there are many observations which suggest that electric polarization and resulting orientation of developing neuroblasts do occur, probably by differential growth of their parts. This is a matter that can be tested experimentally. Differences in electric potential developed within a nerve fiber during the passage of a nervous impulse, the so-called negative variation, are readily measured quantitatively, and electric currents of like intensity are known to be adequate stimuli for nerve fibers. Indeed, if one resting nerve is laid across another and one of the nerves is stimulated, its action current may excite a nervous impulse in the other. It is also known that the more active parts of adult organisms, and in developing embryos regions of more active growth, generate electric currents which are sufficiently strong to serve as an adequate protoplasmic stimulus of more inactive regions.

Taking advantage of these facts and of the further fact that neuroblasts can be dissected out of young vertebrate embryos and their further development in nutrient media watched from moment to moment for as long a time as desired, the way is open to direct experimental approach to the study of the effects of weak electric currents upon the course of growth and differentiation. Ingvar (34), having made such preparations, placed the growing cultures in an electric field and found that the newly formed nerve fibers do orient themselves at right angles to the electric conductors. He summarizes his conclusions as follows:

> The galvanic current has a directing influence upon the *cell and fiber outgrowths in the cultures* so that this occurs almost entirely *along the lines of force* in the galvanic field. Whereas in the control preparations the outgrowth occurs in all directions, cell movements under the influence of a galvanic current take place toward the anode and the cathode. The cell processes growing toward

the anode show morphological differences from those growing toward the cathode. A new biological cell character may in this way be revealed.

If a weak electric current by means of a single conductor is drawn through the culture, the outgrowth of the fibers and cells always takes place *perpendicular to the conductor*. Bok has called attention to the fact that in the living organism the nerve fibers grow out from the spinal cord *perpendicular to the long fiber paths* growing down from the brain stem. Kappers has tried to explain this as a galvanotropic phenomenon. To this observation, an interesting analogy is thus found in tissue cultures. . . . .

As several authors (Hyde, Mathews, Pfeffer) have pointed out, electrical currents flow in developing organisms. The currents successfully employed in our experiments correspond in range in electromotive force with those found in various embryos. From this it may be concluded that electrical forces play a rôle in the formative processes in morphogenesis.

More recently the effect of electric currents upon the rate of regeneration of severed nerves has been studied by Kosaka and Izawa (37). In rabbits, regeneration of severed peripheral nerves is facilitated by the passage of a galvanic current of about 0.1 milliampere down the central stump in the direction from its proximal toward its distal end and regeneration is inhibited by passage of the current through the stump in the reverse direction. After vagotomy in the lower cervical region cell bodies of the ganglion nodosum and of the nucleus ambiguus remain unaltered 48 hours after the operation. The same result is obtained if the electric current is passed downward through the central stump of the nerve, but if the current is passed through the stump in the reverse direction these cell bodies are in pronounced chromatolysis at the end of 48 hours. After 5 days of similar treatment the different effects of descending and ascending electric currents are still more pronounced. In one case the facilitation of peripheral regeneration acts to check the chromatolysis; in the other case the interference with normal regeneration accelerates the chromatolysis.

The bioelectric factors in differentiation are evidently intimately associated with metabolic processes and with the physical properties of interface membranes, their permeabilities, etc. (39).

Fourth, numerous observations have shown a close correlation between the vascularization of particular regions of the embryo and the progress of tissue differentiation within them. Coghill and Moore (17) have carried this correlation further, demonstrating an intimate relationship between the progress of vascularization of the tissues and the development of specific behavior patterns. Studies of the progress of vascularization of different parts of the brain of the rat have recently been published by Craigie (18), who finds a correlation between in-

crease in vascular richness and functional maturity of the several parts. It is also evident that "the importance of the blood supply is greater in connection with the metabolic requirements of functional activity than in connection with those of growth in the nervous tissue—functional metabolism requires a greater blood supply than does growth metabolism."

The fifth factor in differentiation, hormone action, with or without a direct chemotactic effect, is suggested by much observation and experiment, though in all of the cases cited below there is still uncertainty as to whether a given effect is produced by chemical activators (hormones), bioelectric phenomena, or perhaps still other influences. Clearly there is in some of these cases some sort of an action-at-a-distance by which one part does influence another with which it is not in protoplasmic continuity. One of the most interesting of these cases has recently been described by Davenport Hooker (32). A series of experiments on young amphibian embryos in which a segment of the spinal cord was removed, rotated on its long axis and reimplanted gives indication of the existence of an attractive stimulus as a factor in reëstablishing the continuity of the cord; for it is possible to obtain reëstablishment of anatomical continuity between a portion of the cord in its normal position and a segment which has been rotated on its long axis through various arcs up to a complete inversion (180° rotation). The regenerating fibers take a spiral course to effect connection with corresponding regions of the rotated segment.

Wieman (48) also got somewhat similar results after excising a short piece of the spinal cord and then reimplanting it at right angles to its former position. Fibers growing from the proximal stump of the cord entered the (original) anterior end of the displaced segment only, then traversed the piece, and finally made functional connection with the posterior stump of the cord. Fibers grew forward from the posterior stump into the (original) posterior end of the displaced segment. The inherent polarity of the transplanted segment seems to determine the direction of growth of fibers from the cut surfaces of the embryonic cord.

In the normal development of Amblystoma embryos, Coghill (16, p. 103) has observed that in the period just preceding the time when fibers of the olfactory and optic nerves grow inward from their peripheral origins to connect with their cerebral centers there is a pulse of very rapid differentiation in these centers. At about this time there is an increase by actual count of 285 per cent in the number of neuro-

blasts in this part of the brain, an increase about ten times as great as in other parts of the brain. There are obviously three possible interpretations of this correlation between rapid peripheral and central differentiation: 1, it may be an expression of hereditary organization; 2, the peripheral differentiation may, through hormone action or otherwise, influence differentiation in the adjacent parts of the central nervous system; 3, the central differentiation may affect the peripheral differentiation through hormone action, bioelectric factors or otherwise and also exert an attractive influence which draws the ingrowing nerve fibers from the periphery into their respective central terminal nuclei. There is experimental evidence that the second and third of these possibilities are actually realized—not to the exclusion of the first.

Burr (7) has shown that, if the nasal epithelium is removed from a very young Amblystoma larva at a stage before the olfactory fibers have grown from it into the brain, the olfactory organ will not regenerate. Clearly, whatever influence the growing brain may exert on the development of the nasal organ, it is not sufficient to lead to the regeneration of this organ after removal. The converse experiment shows that the developing nasal organ does exert sufficient influence upon the brain to determine whether the olfactory cerebral centers can regenerate after removal (8).

Burr performed two series of operations on young Amblystoma larvae. In the first series he removed one cerebral hemisphere and the overlying peripheral olfactory epithelium at a stage before any olfactory nerve fibers had grown into the brain. In these specimens the cerebral hemisphere did not regenerate, showing that in the absence of the nasal organ the olfactory cerebral centers (which make up the greater part of the cerebral hemispheres of young amphibian tadpoles) cannot be restored after removal. In a second series of similar operations Burr removed one cerebral hemisphere, but left the overlying olfactory epithelium in place, and in these specimens there was complete regeneration of the cerebral hemisphere, showing that some influence from the peripheral olfactory organ is essential for the regeneration of the olfactory centers of the brain.

It is interesting to note that in these last experiments, where the hemisphere was removed but the peripheral olfactory epithelium was left in place, there was some growth of the regenerating cerebral hemisphere before any olfactory fibers had reached the brain; but in those cases in which the olfactory epithelium was also removed this growth did not take place. It may be inferred that the developing olfactory

epithelium produces some soluble substance (hormone) which diffuses through the tissues and activates the cells of the adjacent brain wall to more rapid proliferation, or that differences in bioelectric potential in the growing olfactory organ can act across the very short distance between this organ and the brain and so stimulate growth in the latter, or that the permeability of the tissue has been modified in some other way.

From these experiments it is clear that there is some sort of influence exerted upon the developing brain from the rapidly growing peripheral sensory surface and that this is antecedent to the appearance of protoplasmic connections between these regions. This influence is probably physiologically of much more generalized nature than a true nervous impulse, and indeed it may be some phase of the general physiological gradients or some allied kind of process. These experiments give no evidence of influence exerted in the reverse direction, from brain to nasal organ, but other experiments to be mentioned shortly do suggest some such action in later developmental stages.

The idea has often been expressed that during early development particular muscles or groups of muscles produce specific hormones which exert an attractive or chemotactic influence upon the motor nerve fibers which are at the same time pushing their way out from the central nervous system, so that only those nerve fibers which are in chemical attunement with or which are chemically susceptible to the hormone from a particular muscle will grow toward that muscle. There is some evidence for such a differential preference (20), (21), and this may be a real factor in the nicely balanced chemical equilibrium of the normally developing body, but clearly this specificity, if present, is not very strong, for under some experimental conditions nerves will grow and effect functional connections in most atypical patterns. That the specific affinity of any growing nerve for its own particular end organ is very weak, at least in the earliest stages before tissue differentiation is far advanced, is clear from numberless experiments, as, for instance, those of Burr (10) on transplanted olfactory placodes and Stone (45) on other transplants. The experiments of Stone show that epithelial sensory placodes which normally develop into specific sensory ganglia (lateral line, general cutaneous, etc.) may retain that specificity in whole or in part after transplantation to abnormal positions, or when transplanted so as to replace placodes of other specific type they may within certain limits assume the rôle of the placodes for which they have been interchanged.

The problem of the limits of "self-differentiation" of isolated parts is too involved to be considered in this review, though attention may be called to the fact that isolated parts of the primordial nervous system, if transplanted to an "indifferent" field (chorio-allantois of hen's egg) show a high degree of capacity for further differentiation, as recently shown by Danchakoff and Agassiz (19), Hoadley (31), and many others. That specific hormones of morphogenetic power are formed during these stages of differentiation is not improbable, though we lack actual demonstration of their action.

The more remote effects of endocrine activities involving wide dispersal of the chemical activators through the body fluids are not considered in this review.

A sixth factor in the differentiation of the nervous system is the penetration of nerve fibers into a region previously not so innervated. Many years ago Landacre (38) demonstrated in the catfish (Amiurus) a close correlation between the time and place of appearance of taste buds and the penetration of their nerve supply into their vicinity. Olmsted later tested the significance of this correlation experimentally. In the same fish (40), (41) he showed that, on section of the nerves supplying taste buds on the barblets, the buds disappear by degeneration and resorption (not by dedifferentiation as supposed by some former observers) and that upon regeneration of the nerves the buds reappear by mitosis and differentiation of indifferent cells of the germinative layer of the epithelium. In still later experiments (42), (43) performed on dogs, the taste buds supplied by the chorda tympani were shown to degenerate in the same way after severance of the fibers of this nerve. It may be concluded that differentiation of taste buds, both in embryonic development and in regeneration, is dependent upon contact or close proximity of their specific nerve supply and that upon loss of this innervation the buds disappear by complete degeneration and resorption of their specific receptive cells.

Detwiler (20), (22) finds that in Amblystoma differentiation of sensory neurons is more dependent upon peripheral influence than is that of motor neurons, for the latter appear to be able to develop in the absence of their appropriate musculature under the influence of central correlation fibers. He says that, though afferent neurons suffer reduction after loss or disturbance of their peripheral sensory fields, he obtained no evidence "that the extent of differentiation of motor neurons is in any way under the control of peripheral functional requirements."

Within the central nervous system the entrance of growing nerve

fibers into a new field may be accompanied by notable local activity. Such a field may be growing very slowly, but immediately upon the entrance of a developing nerve tract a pulse of growth is inaugurated which is followed by increase in the size of the part as well as by the rapid maturing of its neuroblasts toward the functional condition. And this increase may arise in very atypical places if fibers grow into them from sense organs which have been transplanted from their normal positions.

The external form of the forebrain of Amblystoma and the sculpturing of the ventricular surfaces have recently been figured (27) and these configurations are similar in all Amphibia with minor variations characteristic of the several species. Some preliminary observations on the embryologic development of these patterns suggest that subsequent to the earlier stages in the plastic development of the neural tube a new morphogenetic factor appears at the time when axons from rapidly differentiating regions penetrate regions of less advanced development. Simultaneously with the penetration of these retarded fields by growing fiber tracts there may appear within them a pulse of local growth. The matter has not yet been investigated in detail, but it seems probable that there is a correlation between these local pulses of growth and the ingrowth of the fibers. Whether the rapid growth of previously inactive regions exerts an attractive influence upon the nerve fibers growing out from other places or whether, on the other hand, penetration of an inactive region by new axons excites it to more rapid growth remains to be determined.

The second alternative is favored by observations of Bok to which reference is made beyond and by Detwiler's experiments (20), (22). The latter author has shown that if a limb is transplanted into the region of the body normally supplied by the 7th, 8th and 9th segments of the spinal cord there is increase in the number of sensory neurons in these spinal cord segments but no increase in the motor neurons. If, however, the 7th, 8th and 9th segments of the cord, which do not normally innervate limbs, are substituted for the 3d, 4th and 5th segments, which normally do so, the transplanted segments effect normal connections with the limb and there is an increase in their motor as well as sensory neurons up to the number normally present in the segments of the limb level. The motor increase may take place in the absence of the limb (24). Evidence is given that this increase in the motor neurons of the transplanted segments is due to the influence of internal fiber tracts normally present at this level and absent from the level of the 7th, 8th

and 9th segments. This evidence receives further support (23) from the experimental reversal end-for-end of the 3d, 4th and 5th segments of the cord, resulting in the development of the normal pattern of spinal cord and peripheral nerves from the operated segments.

It should be borne in mind, however, that Coghill (16) has shown that in Amblystoma larvae of the stages immediately subsequent to those upon which Detwiler operated the 3d, 4th and 5th segments of the cord are in a field of very rapid cell proliferation which seems to be an expression of cephalo-caudal pulses of growth whose underlying causes have not yet been determined, and the matter evidently needs further investigation. These considerations apply also to Detwiler's transplantation experiments already cited (22). The relative part played here by the primary physiological gradients and the influence of ingrowing fiber tracts remains to be determined.

Detwiler's experimental transplantations of limbs to abnormal positions have shown clearly that if the peripheral field normally innervated by a given segment of spinal cord is artificially increased (excessive peripheral loading) there results a notable increase in the number of sensory neurons related with that segment both peripherally and centrally, but the motor neurons of that segment are not obviously affected. The inference is that the morphogenetic influences in the first case come chiefly from the periphery and in the second case chiefly from the central nervous system.

Coghill (16) is one of the few students of the growth of the nervous system who has carefully distinguished in a quantitative way between *proliferation*, or increase in the number of elements by cell division, and *differentiation*, or the progress toward maturity of the individual cells; and he has clearly brought out certain radical differences in the processes involved and in the general physiological significance of these two phases of growth.

It seems probable that during proliferation stages the hereditary factors, the physiological gradients and bioelectric polarities (these last two being in part different aspects of the same processes) and the chemotactic influences are all very potent and that the three last mentioned are not very specific, any region of rapid proliferation acting as a center of local dominance over all other parts within the range of its influence. With the beginning of differentiation of neuroblasts in any part of the nervous system the entire physiological aspect is changed, and this may be the explanation for some of the discrepancies between the results of various observers who have studied the same problems

experimentally. For slight differences in the times at which the experimental operations were performed may give widely divergent results depending on whether the site of operation was at the moment in the phase of proliferation or differentiation.

In every part differentiation goes on alternately with and largely to the exclusion of cell proliferation, for these are independent variables; pulses of first one, then the other appear. Coghill's observations suggest that these pulses tend to begin in the brain and to follow down the length of the central nervous system, taking a course parallel with (and possibly in some way related to) the primordial physiological gradients of the vertebrate body.

Within the general polarity of a region as a whole, as determined during the proliferation stages, each individual neuroblast during the differentiation stage assumes an intrinsic polarity of its own which is active in shaping its further career (29). As its fibrous processes elongate and penetrate ever more remote territory, these outgrowing fiber tracts become new centers of active morphogenetic influence. This influence is manifest long prior to the appearance of true nervous conduction or closed circuits of reflex transmission. At much later stages these transmission mechanisms are further developed through use, a process which must not be confused with these very early differentiations.

The independence of these two processes of differentiation, the earlier and the later, is brought out very clearly by experiments designed to test the mutual influence of the olfactory organ and the cerebral hemisphere. In one of Burr's experiments (9) he transplanted one cerebral hemisphere and the overlying olfactory epithelium in prefunctional stages into the skin of the side of the body. The experiment was performed in two ways: in the first the olfactory epithelium was grafted in at the surface of the body so that after functional maturity it could be stimulated normally; in the second case it was implanted below the skin so as to preclude stimulation in the normal way. In both cases there was further growth of both the olfactory organ and the cerebral hemisphere and they differentiated up to a certain point in nearly normal fashion, save for reduction in size of those regions of the hemisphere normally receiving nerve fibers from lower parts of the brain. The presence of the olfactory organ and its nerve, even in the absence of the possibility of their normal functional activity, ensures the further growth and differentiation of the related cerebral center. We know that the transplanted olfactory placode will grow in the absence of the cerebral hemi-

sphere (45), but whether the transplanted hemisphere will grow at all without the placode or if placed in contact with indifferent ectoderm has not been reported.

In older specimens of Amblystoma and Triton (presumably adult) Weissfeiler (47) removed one or both olfactory bulbs with or without destruction of part of the adjacent cerebral hemisphere and observed the process of regeneration of the olfactory nerve and bulb. The reaction of the tissues in this case is very different from that following Burr's operations on prefunctional stages. Here the physiological effects of actual nervous transmission from the uninjured olfactory epithelium (to be further considered immediately) are doubtless operative. It is reported that after destruction of the olfactory bulb the olfactory nerve does not degenerate, but on the contrary it is the site of a centripetally directed regeneration. Neuroblasts derived from the olfactory mucosa are added to the regenerating olfactory bulb. In the injured hemisphere there is also multiplication of neurons at the site of injury. The penetration of regenerating fibers from the newly formed olfactory bulb facilitates this proliferation and differentiation. The pia mater plays an important rôle in this regenerative process and if this membrane has been destroyed the regeneration is atypical and malformations result.

Finally, we have to consider the formative influence of actual nervous transmission through differentiated nerves as a factor in the growth and elaboration of the nervous system. That the neurons of reflex circuits do undergo further development and elaboration during functional stages, largely as a direct effect of use or practice, has been very difficult to prove, though much theory in physiology, psychology and education has been based upon this assumption. Clear evidence that just this does take place in the very early development of the reflex pattern of Amblystoma has very recently been published by Coghill (15).

The development of the behavior pattern in Amblystoma involves definitely and certainly the growth and differentiation of specific neurons while they function as conductors. In other words, the development of the behavior pattern, which is a process of learning in the broadest sense, is actually development structurally and functionally. Although this is not the place to attempt an application of this most definite and specific anatomical explanation of the development of the behavior pattern to conceptions of psychology, it may be noted that this principle of growth-function fusion in the nerve cell must have significant bearing upon problems of instinct, habit and learning. In so far as psychology has use for a nervous system, it must take account of this growth-function of neurons.

In an interesting series of observations on the development of the cranial nerves and their central tracts Bok (5), (6) has studied the relations of the growth processes to the stimulation currents traversing the nervous pathways already laid down. The general principle is that the passage of nervous impulses through an embryonic nerve tract activates the surrounding tissues, resulting in the origination of new nervous paths arising at right angles to the course of the original functioning tract.

New tracts arise from neuroblasts which lie in the vicinity of tracts which are already functioning. The passage of nervous impulses through the preëxisting tract activates the surrounding field, thus stimulating growth in its undifferentiated cells. The young neuron has at the beginning no contact with the fiber tract that stimulates it. Axons arise from the neuroblasts before their dendrites. Contact with the stimulating fiber tract is later effected by growth of the dendrites from the neuroblasts and of collateral branches from the fibers of the tract. When nervous impulses pass through an unmyelinated embryonic fiber tract, energy (probably electric in nature, that is, dissociated ions) radiates outward at right angles to the course of the tract, thus polarizing the surrounding field. The neuroblasts lying in this polarized field when excited to development grow in a direction determined by the lines of the activating force, that is, at right angles to the preëxisting tract.

Many illustrations of this principle are given by Bok. The mechanism is probably a series of bioelectric phenomena similar in principle to those acting in the primary axial gradients of younger embryos in determining the direction of growth of the first neuroblasts to differentiate.

The facts reported by Bok from fixed preparations are objective; the explanation given is theoretic. The hypothesis of stimulogenous fibrillation may be formulated in these words: Frequently repeated excitation currents in a nervous pathway are causes leading to the differentiation of neuroblasts in contiguous fields and the developing nerve fibers tend to take courses at right angles to the direction of the exciting nervous currents. In the earlier developmental stages it is evident that the growth energies of the fibers may activate the surrounding tissue long before actual nervous circuits are functionally closed and, in fact, some of the phenomena described by Bok are evidently in prefunctional stages.

In a study of atypical development of the cerebral cortex Black (3) cites Roux and Bechterew in support of the idea that in the nervous

system the completion of nervous circuits marks the "critical point" between Roux's period of self differentiation and the period of dependent differentiation. But it should be borne in mind that there are apparently two critical periods from this point of view, first the stage marked by the activation of relatively retarded regions by ingrowth of new nerve fibers, and second the further activation of more or less differentiated tissue by use during the passage of nervous impulses through pathways after these have attained functional connection in closed circuits. The exact expression in growth of the first of these influences —whether by proliferation or differentiation or in both of these ways— has not been determined; the second influence is apparently wholly expressed as differentiation.

Again reverting to the phylogenetic problems with which this survey began, mention should be made of Kappers' principle of neurobiotaxis. This principle was enunciated in 1907 and around it an extensive literature has since grown up, emanating chiefly from the Dutch school at Amsterdam. In the course of the phylogenetic differentiation of the nervous system there have occurred more or less extensive migrations of the cell bodies of various functional systems of neurons. A very large number of cases of this sort have been recorded, especially among the motor nuclei of the cranial nerves (4), (33), (35), (36). The principle may be expressed in these propositions:

If nervous impulses are discharged into the nervous system from different directions, there follows an outgrowth of the chief dendrites and eventually a migration of the cell bodies of the neurons which receive these excitations in the direction from which they receive their maximum stimulation. This implies that, if in the course of phylogeny there has been a change in the relative functional importance of the different functional paths which converge into a particular nerve center, the cell bodies of that center will move from the first position toward the source of the newly acquired stronger nervous currents. Thus, if a species with poorly developed organs of taste develops in the course of phylogeny into a species with highly developed gustatory apparatus, the motor nuclei concerned with the taste reflexes (mastication and swallowing) will move toward the gustatory centers.

This approximation of two functional systems by outgrowth of dendrites and migration of cell bodies will occur only in case the systems in question are habitually activated simultaneously or in close succession.

Thus, according to Kappers, dendrites and cell bodies tend to move

toward the source of chief excitation, that is, against the current of the normal discharge, while axons tend to grow out in the direction of normal conduction. This arrangement evidently has a certain adaptive value, for axons are structurally better conductors than are dendrites and the shortening of dendrites improves the conductivity of the reflex arc. Kappers (35) and Bok (5), (6) have elaborated hypotheses in which the mechanisms of neurobiotaxis and stimulogenous fibrillation are explained in terms of bioelectric polarities. Child (13), (14) in the course of his analysis of the phenomena of histological differentiation in the nervous system has also expressed his results in terms of bioelectric influences in a form differing in some respects from those of Kappers and Bok but in general agreement with the broad lines of their method of approach to the problem.

These rearrangements of nerve cell bodies are in the first instance functionally determined during individual development. They may later be incorporated into the hereditary organization (by natural selection, "organic selection," or otherwise), or they may in every new generation be functionally (epigenetically) determined during development. We do not know.

In summary, the three stages in the development of the nervous system to which attention is here especially directed are: 1. Cell proliferation, or increase in number of cells at a stage when none of the cells have become sufficiently matured or differentiated to function normally. 2. Cell differentiation, or the growth to functional efficiency of the cells and the development of their diverse functional patterns and connections. 3. The influence upon further development of actual functional transmission of nervous impulses through the reflex circuits and the further complication of these circuits by the appearance of collateral pathways. Coincident with this last is the rise of the centers of higher (that is, more complex) correlation.

In this review no attempt has been made to cite all of the important recent literature dealing with these complex and recondite processes of ontogeny and phylogeny. Illustrative cases only have been selected as samples of the trend of current investigation. It must not be assumed that any of the agencies here enumerated are alone competent to account for the complex history of early morphogenesis, nor do all of these in the aggregate comprise the total number of formative influences. These are doubtless real elements among many others in a very intricate system of physiological gradients, bioelectric fields, chemical activators and transportative mechanisms which make up the life

pattern of the growing embryo, a dynamic pattern which changes with kaleidoscopic rapidity during the whole of the growth period.

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